American Journal of Veterinary Research

A Publication of the
American Veterinary Medical Association

VOL. V OCTOBER, 1944 NO. 17

Consecutive order of appearance, rather than identity with any given volume, is the plan adopted for numbering the issues of the American Journal of Veterinary Research. Following are the volume and issue numbers published to date.

VOL. I, No. 1—OCTOBER, 1940

VOL. II, No. 2—JANUARY, 1941
VOL. II, No. 3—APRIL, 1941
VOL. II, No. 4—JULY, 1941
VOL. II, No. 5—OCTOBER, 1941

VOL. III, No. 6—JANUARY, 1942
VOL. III, No. 7—APRIL, 1942
VOL. III, No. 8—JULY, 1942
VOL. III, No. 9—OCTOBER, 1942

VOL. IV, No. 10—JANUARY, 1943
VOL. IV, No. 11—APRIL, 1943
VOL. IV, No. 12—JULY, 1943
VOL. IV, No. 13—OCTOBER, 1943

VOL. V, No. 14—JANUARY, 1944
VOL. V, No. 15—APRIL, 1944
VOL. V, No. 16—JULY, 1944
VOL. V, No. 17—OCTOBER, 1944

COPYRIGHT 1944 BY AMERICAN VETERINARY MEDICAL ASSOCIATION

Printed in the U. S. A.

Editorial Staff

L. A. MERILLAT and J. G. HARDENBERGH, *Editors*
HELEN S. BAYLESS, *Assistant Editor*

Committee on Journal

W. A. HAGAN, JAMES FARQUHARSON, B. T. SIMMS

Research Council

Anatomy and Histology

H. L. FOUST, D.V.M., Professor of Veterinary Anatomy, Division of Veterinary Medicine, Iowa State College, Ames. (1946).

Bacteriology (Immunology and Biologic Therapy)

EDWARD RECORDS, V.M.D., Research Professor of Veterinary Science, University of Nevada, Reno. (1947).

Biochemistry and Animal Nutrition

GEORGE H. HART, V.M.D., M.D., Head, Division of Animal Husbandry, University of California, Davis. (1947).

Large Animal Medicine

JAMES FARQUHARSON, D.V.M., Professor of Surgery and Clinics, Division of Veterinary Medicine, Colorado State College, Ft. Collins. (1947).

Large Animal Surgery

W. F. GUARD, D.V.M., Professor of Surgery and Obstetrics, College of Veterinary Medicine, The Ohio State University, Columbus. (1945).

Parasitology

E. W. PRICE, D.V.M., M.S., Ph.D, Assistant Chief, Zoölogical Division, Bureau of Animal Industry, Beltsville Research Center, Beltsville, Md. (1945).

Pathology

E. T. HALLMAN, D.V.M. (*Chairman, Research Council*), Professor of Pathology, Division of Veterinary Science, Michigan State College, East Lansing. (1947).

Physiology and Pharmacology

H. H. DUKES, B.S., D.V.M., M.S. (*Secretary, Research Council*), Professor of Veterinary Physiology, New York State Veterinary College, Cornell University, Ithaca. (1947).

Poultry Pathology

C. A. BRANDLY, D.V.M., 25 Shattuck St., Boston. (1945).

Small Animal Medicine

M. L. MORRIS, D.V.M., Practitioner, New Brunswick, N. J. (1946).

Small Animal Surgery

C. F. SCHLOTTHAUER, D.V.M., Associate Professor, Division of Experimental Medicine, Mayo Foundation, Rochester, N. Y. (1946).

Veterinary Hygiene

CHAS. A. MITCHELL, Animal Disease Research Institute, Hull, Quebec. (1946).

Virus Diseases

R. A. KELSER, D.V.M., A.M., Ph.D. (*Vice-Chairman, Research Council*), Brigadier General, United States Army; Chief, Veterinary Division, Office of the Surgeon General, War Department, Washington, D. C. (1946).

X-Ray

M. A. EMMERSON, D.V.M., M.S., Dr. med. vet., Professor of Veterinary Surgery and Obstetrics, and Radiologist, School of Veterinary Medicine, University of Pennsylvania, Philadelphia. (1945).

Member-at-Large

E. A. WATSON, V.S., Animal Diseases Research Institute, Hull, Quebec; Division of Animal Pathology, Department of Agriculture, Dominion of Canada. (1945).

CONTENTS

Action of Chemical and Physical Agents on the Virus of Equine Infectious Anemia —C. D. Stein, O. L. Osteen, L. O. Mott, and M. S. Shahan..... 291

Experimental Infections with Eimeria Bovis in Calves—Datus M. Hammond, Leonard Reid Davis and George W. Bowman..... 303

Calcium Boro-Gluconate-Saccharate: Preparation, Toxicity and Effect on Serum Calcium Level in Cows—A. Appleby..... 312

Studies on Bovine Mastitis I. A Note on Clavacin Therapy in Bovine Mastitis— E. H. Peterson and Robert Graham..... 316

Isolation of the Bacillus Anthracis from Spinose Ear Ticks Ornithodorus Megnini —George W. Stiles..... 318

Studies on Johnin V. Producing and Standardizing a Potent Product—Howard W. Johnson 320

Brucellosis of Swine II. Eradication by Blood Test and Segregation—H. S. Cameron and P. A. Carlson..... 329

Brucellosis in Swine III. Studies on the Diagnostic Titer in the Individual—H. S. Cameron and P. A. Carlson..... 333

Urine Excretion by Boars—W. W. Green..... 337

Sulfur Prophylaxis of Coccidiosis of Feeder Lambs—John F. Christensen..... 341

Published quarterly at 600 S. Michigan Ave., Chicago, Ill., by the American Veterinary Medical Association. Entered as Second-Class Matter January 31, 1941, at the Post Office at Chicago, Illinois, under the Act of March 3, 1879. Subscription price: $4.00 per year for United States, Canada, and countries affiliated with the Pan American Postal Union; foreign countries, $4.30. Members of the American Veterinary Medical Association may subscribe to the American Journal of Veterinary Research for $2.50 per year added to the annual dues of $7.00. Single copies $1.25. Address all correspondence to American Veterinary Medical Association.

American Journal of Veterinary Research

Volume V OCTOBER, 1944 Number 17

Action of Chemical and Physical Agents on the Virus of Equine Infectious Anemia

C. D. STEIN, V.M.D., O. L. OSTEEN, D.V.M., L. O. MOTT, D.V.M., and M. S. SHAHAN, D.V.M.
Washington, D. C.

STUDIES on the nature and properties of the virus of equine infectious anemia have been made by a number of investigators. Although considerable information has thus been ascertained, the exact nature of the virus still remains a matter of discussion. Most investigators agree that it is ultramicroscopic, capable of passing through filters that retain bacteria, and that it is specific for solipeds. It has not been cultivated *in vitro* and no convincing evidence has been reported to indicate that it is able, consistently, to reproduce itself in animals other than the equine species. Our studies on 9 different strains, isolated from widely separate areas, indicate that its virulence is variable.

According to Kral, Macek, and Sobra,[1] the virus is specific and shows strong cellular hemotropism. These authors state that it is associated with albuminous materials; is not ultrafiltrable, and possesses considerable resistance against ordinary disinfectants. Balozet,[2] who carried out filtration experiments with the virus, using collodion membranes of different porosities, reported that the diameter of the virus particles is probably between 18 and 50 m μ. According to Von Mócsy,[3] the virus appears to be attached to blood proteins or is a protein in itself. He further reported that in the blood of infected animals, the globulin and albumen are infective, whereas the dialysate is noninfective, and that large doses of x-ray, lethal to normal cells, produce no action on the virus. Heath[4] reported that the virus is not dialysable and that it is either not present in the globulin fraction of virulent plasma or is destroyed by the ammonium sulfate used to separate this fraction from the serum.

A considerable amount of work on the resistance of the virus to chemical disinfectants and physical agents has been carried out by various investigators with somewhat conflicting results.

In experiments on the resistance of the virus to phenol, Luhrs[5] reported that 4.0 per cent phenol destroys the virus in less than one hour. The Japanese Commission[6] found that 2.0 per cent destroys the virus in one hour, whereas Balles,[7] using the same concentration, found the virus resistant for more than twenty-four hours. Heiermann[8] found that the virus is killed in four to six weeks with 0.5 per cent concentration and in eight weeks with 0.1 per cent. On the other hand, Frohner and Bierbaum[9] reported that it requires ninety days to destroy the virus with a concentration of 0.1 per cent. Semenov and Oldenborger[10] reported that virulent serum preserved with 0.5 per cent phenol is still infective but attenuated after six months. Balozet[11] considers phenol to be weakly destructive to the virus and believes the virus contained in serum to be more resistant against phenol than that in spleen tissue. He found that 0.1 per cent phenol has no effect on virulent serum in twenty-four hours but destroys the virus in four months ten days, whereas the same concentration of phenol in a 1 to 10 saline suspension of

From the Pathological Division, Bureau of Animal Industry, Agricultural Research Administration, Washington, D. C.

spleen tissue destroys the virus in sixteen days at 16 to 18 C.

In experiments with formalin, Von Mócsy[12] showed that the blood or tissues containing virus lose their virulence after thirty-eight to seventy-four days' contact with 0.3 to 0.5 per cent formalin at refrigerator temperature, whereas serum retains its virulence after six days' contact with 0.3 per cent formalin. Heath[4] reported that the virus in the red blood cells and plasma is destroyed by the addition of 0.025 per cent formalin.

In studies on the effect of other chemical agents, the Japanese Commission[6] found that virus is destroyed by the addition of 2.5 to 5.0 per cent taurocholate of soda but is not destroyed by 3.0 per cent cresol in twenty-four hours; chloroform and toluol were found to have little effect. Kral, Macek, and Sobra[1] found that virus in serum is destroyed in three months when 0.5 per cent phenol or equal parts of 0.6 per cent tricresol and ether are used as a preservative. Balozet[11] showed that virus in serum is not destroyed by 3.0 per cent sulfuric ether or 1.5 per cent chloroform in four and one-half months. He also showed that the addition of 0.1 per cent of a 10.0 per cent solution of sodium fluoride to virulent serum fails to destroy virus in seven months, that saponin in concentration of 1.0 per cent destroys it in less than twenty-four hours, that 50.0 per cent glycerin acts slowly and progressively on the virus contained in virulent serum, producing attenuation after four months' contact and destruction after seven months' contact. Balozet[13] showed that some modification occurs in twenty-four hours when virus is mixed with sterile bile in proportions of 1 to 10 up to 1 to 2. Troitskit and Klimov[14] showed that virus *in vitro* is inactivated after exposure to gastric juice for one hour. Martin[15] has shown that virus is adsorbable on aluminum hydroxide and that the complex so formed is not modified by bile but is attenuated by formalin in certain proportions.

In experiments on the resistance of virus to physical agents, Hempel[16] was unable to inactivate it by heating to 58 C. for one hour but Carré and Vallée[17] found that heating at this temperature for one hour destroys it. The Japanese Commission[6] found that heating for one hour at 60 C. renders it noninfective and that direct exposure to sunlight at 49 C. destroys it in two hours. This group of investigators also showed that it would retain its virulence for four months in freezing temperatures often as low as —8 to —9 C. and that it remained virulent for six months when stored at less than 0 C. They also found that it retained its virulence when desiccated in vacuum at room temperature and held for ten days but not after having been stored for seven months. Van Es, Harris, and Schalk[18] found that exposure to severe freezing weather for one month (33 to —26 F.) did not affect the activity of virulent blood. Semenov and Oldenborger[10] reported that virus remained virulent for five months at room temperature.

PURPOSE OF EXPERIMENTS

It is a well-recognized fact that infectious anemia may occur in horses in an inapparent or subclinical form and that animals that have had an acute attack may make an apparent recovery but still harbor the virus for years. Since such horses appear normal and no reliable laboratory diagnostic test is available for the detection of carriers, infected horses may pass physical examinations and thus gain admission to institutions preparing biological products. The danger of disseminating the disease through the use of antiserums or vaccines prepared from such animals is obvious and presents a problem of great importance. A number of instances are on record that outbreaks of the disease in horses have occurred in biological institutions, both in the United States and in foreign countries.

The experiments recorded in this paper were, therefore, carried out primarily to obtain information that would assist in safeguarding veterinary and human biological products prepared from horses from possible contamination with the virus of equine infectious anemia. Special attention was given to studies on the effects, on the

virus, of chemical and other agents commonly used in the preparation of antiserums and vaccines. It was believed that information on the modification or attenuation of the virus for producing a vaccine against the malady might also be obtained.

MATERIALS AND METHODS IN GENERAL

Since the pathogenicity of the virus is variable, only highly virulent strains were used in these experiments except where stated. In some instances, virulent blood serum from several active cases was pooled before treatment.

Virulent blood, blood serum, or tissues collected from active cases of infectious anemia were subjected to chemical agents, heat, cold, freezing, desiccation, evaporation in vacuum, fractionation by the ammonium sulfate method, and dialysis, to determine the resistance of the virus to these processes. Untreated representative samples of each lot of virus-containing material used in the various experiments were reserved for virulence control tests, if necessary.

The virus-containing materials, after treatment by different methods for varying periods of time, were tested for virulence by subcutaneous inoculation of large amounts into normal horses. The susceptibility of those horses that failed to develop infectious anemia after being injected with the treated material was checked by subcutaneous injection of known infectious anemia virus. The susceptible horses used in these experiments were of light type, 900 to 1,100 lbs., and were held under observation in screened isolation stables provided with concrete floors and screened vestibules. As far as can be determined, none of the normal horses had had any previous contact with horses affected with infectious anemia. Temperatures of all test animals were recorded at least twice daily. Special precautions were taken to exclude all possibility of accidental transmission.

ACTION OF CHEMICALS

Phenol, Merthiolate and Phenol, Phenyl Mercury Acetate and Phenol, and Crystal Violet and Phenol.—The blood serum used in these experiments was obtained from an acute case of infectious anemia, three days previous to death. The serum, after being separated from the blood clot, was filtered through a No. 8 Mandler filter candle and divided into 5 lots.

Lot 1 was untreated and held under refrigeration at 5 C. for use as a control. Lot 2 was placed in an Erlenmeyer flask and merthiolate (ethyl mercuric thiosalicylate) and phenol were added slowly during constant agitation to give a concentration of 1:10,000 and 1:400, respectively. After the reagents were added, the treated serum was agitated for several minutes to insure a thorough mixture. The treated serum was then stored under refrigeration at 5 C. for thirty days. Lots 3, 4, and 5 were handled in the same manner as lot 2 but in lot 3 a mixture of phenyl mercury acetate and phenol was added to give a concentration of 1:10,000 and 1:400, respectively; in lot 4, a mixture of crystal violet and phenol to give a concentration of 0.1 per cent and 0.05 per cent, respectively; and in lot 5, enough phenol to make a concentration of 0.5 per cent.

Each of the five lots, after being held for thirty days at 5 C., was inoculated subcutaneously in 100-cc. amounts into a normal horse. The horses that received lots 1 and 2 developed infectious anemia. Those that received lots 3, 4, and 5 remained well for 104 days and, when subsequently exposed to a virus of known virulence, developed the disease.

This test indicates that the combined action of merthiolate 1:10,000 and phenol 1:400 failed to destroy the virus in thirty days, whereas the action of mercury acetate 1:10,000 in combination with phenol 1:400, of crystal violet 0.1 per cent in combination with phenol 0.05 per cent, and of 0.5 per cent phenol destroyed the virus in thirty days at 5 C.

Formalin, 0.1 per cent and 0.2 per cent.—In this experiment, pooled, unfiltered blood serum, collected from 5 horses infected with infectious anemia, was divided into 3 lots. Lot 1 was not treated with formalin but was used as a control. Lot 2 was treated with formaldehyde solution U.S.P. (formalin) to make a concentration of 0.1 per cent, and lot 3 was treated with formalin to make a concentration of 0.2 per cent. All 3 lots were stored in the refrigerator at 5 C. Each lot, after being held for thirty-three days at 5 C., was inoculated subcutaneously in 50-cc. amounts into a healthy horse. The horse that re-

ceived the untreated serum developed the disease in fourteen days whereas the horses injected with 0.1 per cent and 0.2 per cent formalin remained well for ninety-two days. The susceptibility of the horses injected with the formalin treated lots was subsequently confirmed by exposure to a virus of known virulence.

The results of this experiment indicate that the action of 0.1 per cent or 0.2 per cent formalin for thirty-three days at 5 C. inactivated the virus contained in blood serum.

Formalin, 0.4 per cent.—Experiment 1.—The brain tissue used in this experiment was collected aseptically from 2 horses affected with infectious anemia. It was macerated thoroughly and sufficient physiologic salt solution (normal saline solution) to make a 10.0 per cent suspension was added. After being milled for several hours, the suspension was divided into 2 lots. Lot 1 was untreated and placed in storage at 5 C. for use as a control. Lot 2 was placed in a large, tightly corked container to which sufficient formalin was added to make 0.4 per cent by volume. After being thoroughly mixed, the formalized brain suspension was stored in the refrigerator at 5 C. and was agitated for several minutes daily to insure a thorough mixture of the formalin with the brain emulsion.

After being held at 5 C. for thirteen days, each lot was inoculated subcutaneously in 100-cc. amounts into a normal horse. The horse receiving the untreated material developed typical symptoms of acute infectious anemia on the tenth day, whereas the horse injected with 0.4 per cent formalin did not develop symptoms of the disease until forty days had passed. The results indicate that the action of 0.4 per cent formalin for thirteen days apparently modified or attenuated the virus so as to prolong the incubation period but did not destroy it.

Experiment 2.—The brain tissue used in this experiment was collected from a horse affected with infectious anemia. It was processed in the same way as that used in experiment 1, except that lot 2 was treated with 0.4 percent formalin for thirty-two days. Each lot was then injected subcutaneously in 100-cc. amounts into a normal horse. The horse injected with the untreated portion developed typical acute symptoms of infectious anemia in nine days, whereas the horse treated with 0.4 per cent formalin for thirty-two days remained well for 150 days. The susceptibility of this horse was subsequently confirmed by exposure to virus of known virulence.

The results indicate that the action of 0.4 per cent formalin for thirty-two days destroyed the virus contained in 10.0 per cent saline brain suspension.

Experiment 3.—The brain tissue used in this experiment was collected from a horse affected with infectious anemia. The suspension of brain material was prepared in the same way as that used in experiment 1 but in the third experiment there was no control sample. Four normal horses were each inoculated subcutaneously with 100-cc. of the material treated thirty days with 0.4 per cent formalin and they remained normal for sixty days. The susceptibility of the test animals was subsequently confirmed by exposure to known virus. This experiment indicates that the action of 0.4 per cent formalin for at least thirty days destroyed the virus contained in 10.0 per cent saline suspension of brain tissue.

One per cent Chloroform.—The brain tissue used in this experiment was collected aseptically from a horse affected with infectious anemia and destroyed after an acute reaction. The tissue was thoroughly macerated by passing through a sterile meat grinder, grinding in a mortar, and finally passing through a sieve composed of fine mesh wire screen. Sufficient normal saline was then added to make a 33.3 per cent saline suspension which was divided into 4 lots.

Lot 1 was untreated and stored at 5 C. for sixty days. In lot 2, sufficient chloroform was added to make 1.0 per cent by volume. The mixture was thoroughly agitated, then stored at 5 C. for sixty days and thoroughly agitated daily during the period of storage. Lot 3 was untreated

and stored at 5 C. for ninety days. In lot 4, sufficient chloroform was added to make 1.0 per cent by volume. The mixture was thoroughly agitated, then stored in the refrigerator at 5 C. for ninety days and during the period of storage agitated daily. Each was inoculated subcutaneously in 50-cc. amounts into a normal horse. The horses inoculated with the first 3 lots developed typical infectious anemia, whereas the horse inoculated with lot 4 failed to develop the disease in 102 days. The susceptibility of the last mentioned horse was later confirmed by exposure to a virus of known virulence.

Since the period of incubation in the horse receiving lot 2 was forty-five days, it appears that in this instance, although the virus was not destroyed, it was attenuated or modified so as to prolong the period of incubation and produce a chronic case of the disease.

This test also indicates that the virus contained in a 33.3 per cent saline suspension of brain tissue was killed in ninety days by the action of 1.0 per cent chloroform.

Phenol and Ether Combined.—The blood serum used in this experiment was obtained from an acute case of experimentally induced infectious anemia. The blood was collected in large tubes and allowed to clot. The supernatant serum was then removed for treatment with phenol and ether and divided into 5 lots.

Lot 1 was untreated and stored at 5 C. for thirty days for use as a control. Lot 2 was mixed with sufficient phenol and ether to contain 0.5 per cent of each by volume. After being thoroughly agitated, it was placed in storage at 5 C. for thirty days. Lot 3 was untreated and stored at 5 C. for sixty days for use as a control. Lots 4 and 5 were mixed with sufficient phenol and ether so that the former lot contained 0.25 per cent of each by volume and the latter, 0.35 per cent of each by volume. After these two lots were thoroughly agitated, they were placed in storage at 5 C. for sixty days.

Each lot was inoculated subcutaneously in 100-cc. amounts into a normal horse. All the horses except the one injected with lot 2 developed typical symptoms of infectious anemia. The last mentioned horse failed to develop symptoms of infectious anemia in ninety-four days. The susceptibility of this animal was subsequently confirmed by exposure to a virus of known virulence.

The results of this test indicate that 0.5 per cent phenol and 0.5 per cent ether inactivated the virus contained in the serum in thirty days but that 0.25 per cent phenol and 0.25 per cent ether or 0.35 per cent phenol and 0.35 per cent ether failed to inactivate the virus in sixty days. However, since the 2 horses inoculated with the untreated samples developed the disease in the acute, fatal form, and those treated with the combination of 0.25 per cent phenol and ether for sixty days and the combination of 0.35 per cent phenol and ether for sixty days had a longer incubation period and developed the disease in the chronic form, it appears that the action of phenol and ether in these concentrations may have modified or attenuated the virus to some extent.

EFFECT OF PHYSICAL AGENTS

Heat.—Experiment 1.—To determine the effect of heat on the virus in blood serum from carriers of infectious anemia, serum from 3 horses affected with the inapparent form of the disease was pooled and divided into 2 lots. Lot 1 was not heated but was held as a sample for checking the virulence of the serum. Lot 2 was heated in a water bath at 58 C. for thirty minutes, the temperature of which was controlled by thermometers. The flask containing the serum was agitated by hand from time to time during the heating process.

The samples were tested for the presence of virus by inoculating 450-cc. of each lot subcutaneously into a susceptible horse. The horse inoculated with the unheated sample developed symptoms of infectious anemia after an incubation period of twenty-three days. The horse inoculated with the heated sample remained normal for 160 days but when exposed to a known strain of virus, developed an acute, fatal

form of the disease in ten days. In this instance, the virus in the blood serum of carriers was destroyed in thirty minutes at 58 C.

Experiment 2.—Pooled, virulent blood serum collected from 2 horses affected with acute infectious anemia was divided into 5 lots. Lot 1 was not heated but was reserved for use as a control. Lots 2 and 3 were subjected to 58 and 60 C., respectively, for thirty minutes in a water bath. Lots 4 and 5 were subjected to 58 and 60 C., respectively, for sixty minutes in a water bath. As in experiment 1, the temperature of the water bath was controlled by thermometers and the serum was agitated occasionally by shaking during the period of heating.

Each of the 5 lots was tested for the presence of virus by the subcutaneous inoculation of 150-cc. into a susceptible horse. All the horses except the 1 injected with lot 5 developed typical symptoms of infectious anemia. The nonreacting horse, after remaining normal for 128 days, was subsequently inoculated with a virus of known virulence. The animal developed symptoms of the disease in nine days and died in twenty-three days.

This result indicated that heating at 60 C. for sixty minutes destroyed virus in the blood serum from acute cases. However, the horses injected with lots 1, 2, and 3 developed symptoms of the disease in twelve to nineteen days, whereas the horse injected with lot 4 did not develop symptoms of the disease until ninety-four days after inoculation thus indicating that the virus in this instance had been modified by heat so as to prolong the incubation period.

Experiment 3.—In view of the conflicting results obtained in experiments 1 and 2, a third heating experiment was carried out. In this experiment, the serum was heated by a special apparatus known as a "serum pasteurizer," devised for use in biological establishments. This apparatus was equipped with automatic temperature control and circulating devices to assure continuous mixing of the serum during the heating period, thus making certain the uniform exposure to heat of the virus contained in the serum.

Virulent blood serum collected from one horse (a symptomless carrier) affected with chronic infectious anemia was divided into 5 lots. Lots 1 and 2 were not heated but were reserved for use as controls. Lots 3, 4, and 5 were heated in the pasteurizer for one hour at temperatures ranging from 57 to 58 C., 58 to 59 C., and 59 to 60 C., respectively. Each lot was tested for the presence of living virus by subcutaneous inoculation into a susceptible horse.

Those horses receiving the unheated portions promptly developed infectious anemia, whereas those receiving the heated portions failed to develop symptoms of the disease. Susceptibility to infectious anemia of the horses that failed to develop the disease was confirmed by further exposure to a known strain of virus.

In this experiment, the virus in blood serum from a chronic carrier was destroyed by heating for sixty minutes at 57 to 58 C., 58 to 59 C., and 59 to 60 C.

It is apparent from these 3 experiments that virus in the serum from latent carrier animals (experiments 1 and 3) is more readily destroyed than is virus in the serum from acute clinical cases (experiment 2). It may be assumed, even though comprehensive comparative titrations were not made, that serum from quiescent or occult cases of equine infectious anemia contains virus in smaller amounts or possibly in less pathogenic form than does serum from acutely sick animals. On the other hand, the results of experiment 3 may be taken to support the generally conceded superiority of automatically controlled heating apparatus.

As a whole, the results obtained in the 3 heating experiments indicate that equine serum should be heated for at least an hour at a temperature of at least 58 C. and preferably higher, to insure the absence of active infectious anemia virus. The well-known toxic serum that commonly results from heating for an hour at temperatures much above 59 or 60 C. is reason enough for provision of well-controlled,

automatic pasteurizing devices such as are now required in licensed serum-producing plants.

Cold Storage.—Fifty cc. of serum from an acute case of infectious anemia, held for ninety-three days at 5 C., was injected subcutaneously into a normal horse which developed symptoms of the disease in eleven days.

Fifty cc. of 33.3 per cent saline suspension of brain tissue from an acute case, held for ninety days at 5 C., was injected subcutaneously into a normal horse which developed symptoms of infectious anemia in nine days.

One hundred cc. of defibrinated blood from a chronic case, containing 20.0 per cent buffered glycerin solution* with a pH of 7.4 to 8.0 was held for fifteen months at 5 C. The product was injected subcutaneously into a normal horse which developed symptoms of infectious anemia in thirty-four days.

Seventy-five cc. of defibrinated blood from an acute case, containing 20.0 per cent buffered glycerin solution and held for seven years at 5 C., was injected subcutaneously into a normal horse which remained normal for seventy-four days. The susceptibility of this animal was subsequently confirmed by exposure to a virus of known virulence.

These results indicate that virus retains its virulence in serum for at least ninety-three days and in brain tissue for at least ninety days when kept at 5 C. They also indicate that defibrinated blood containing 20.0 per cent buffered glycerin solution was still virulent after fifteen months' storage but not after 7 years' storage at 5 C.

Freezing.—Virus contained in frozen spleen tissue stored for thirteen and one-half months at —7 to —12 C. was found to be fully virulent when inoculated into a susceptible horse. A 50-cc. dose of a 10.0 per cent saline suspension of this material, when inoculated subcutaneously into a normal horse, produced a typical acute reaction in eight days and death in twenty-four days.

*Glycerin phosphate solution—distilled water 500 cc., glycerin 500 cc., and KH_2PO_4—2.5 Gm.

However, the virus which was active in frozen spleen tissue after thirteen and one-half months at —7 to —12 C. was inactive in the same tissue after twenty-two months eight days' storage at the same temperature as shown by the following: Fifty cc. of the suspension, prepared from spleen, stored for the longer period was injected into a normal horse which remained normal for sixty-three days. The susceptibility of this animal was later confirmed by exposure to a virus of known virulence.

Desiccation.—Spleen and lymph glands, collected from an infected horse at autopsy, were emulsified and spread in very thin layers over sterile Petri dishes which were placed in a vacuum desiccator containing calcium chloride and held at 5 C. After desiccation the hard, dried, brittle layer of material was removed at different times from the Petri dishes, pulverized, and divided into 5 lots.

Lot 1 was removed after two weeks, lot 2 after three weeks, lot 3 after four weeks, lot 4 after nine months ten days, and lot 5 at the same time as lot 4. Lot 5, however, was stored in a tightly corked vial at 5 C. for five years one month.

Of each lot of the powdered spleen and lymph gland, except lot 5, 2.4 Gm. was emulsified in 20 cc. of physiologic salt solution and inoculated subcutaneously into a normal horse to test for the presence of virus. In lot 5, 6 Gm. in 50 cc. of physiologic salt solution was injected. Each of the lots except lot 5 produced symptoms of infectious anemia in the normal test horses a short time after injection. The horse inoculated with lot 5 remained normal for seventy-four days. The susceptibility of this horse was subsequently confirmed by exposure to a virus of known virulence.

The results of this experiment indicate that virus contained in desiccated tissue stored as long as nine months ten days at 5 C. in a vacuum desiccating jar remained virulent but that the same material, when stored for five years one month at 5 C. in a tightly corked container, was no longer virulent.

Dehydration Under High Vacuum While in a Frozen State (Lyophilization).—Whole

blood, aseptically collected from a subacute case of infectious anemia, was divided into 2 lots. Lot 1 was untreated and was held as a control sample for testing the virulence of the virus. Lot 2 was pipetted in 5 cc. amounts into a number of sterile ampules. The material in the ampules was first rapidly frozen at a very low temperature and then desiccated under high vacuum (lyophilization). The dehydrated blood was then flame-sealed *in vacuo* in the glass ampules, and stored at room temperature (exposed to light) until tested for infectivity.

Two days after dehydration, a sample of each lot was tested for infectivity. A normal horse was inoculated subcutaneously with 10 cc. of whole blood from lot 1 and another normal horse with the contents of 2 ampules of the dehydrated blood from lot 2 suspended in 20 cc. of distilled water. Both horses developed symptoms of infectious anemia on the twelfth day. This indicated that the process of lyophilization had not materially affected the stability of the virus.

After sixteen months' storage at room temperature in the presence of light, 2 samples of lot 2, were each suspended in 20 cc. of physiologic salt solution and inoculated subcutaneously into each of 2 horses. Both of them developed symptoms of infectious anemia on the nineteenth day. This indicated that the virus in the dehydrated blood retained its virulence for at least sixteen months *in vacuo* at room temperature.

Effect of Fractionation and Dialysis on Virus in Virulent Serum

Since the fractionation of serum by salting with ammonium sulfate followed by dialysis is commonly employed by biological concerns in the refinement of antiserums prepared from horse blood, the following experiments were carried out to determine what effect, if any, this treatment might have on virus contained in blood serum. It was believed that some information could also be obtained as to what serum fraction, if any, contained the virus.

The fractionation of serum by means of the ammonium sulfate salting out method is carried out by biological concerns to obtain the pseudoglobulin fraction in pure state. However, considerable preliminary treatment of the serum is required before this can be accomplished. It is first necessary to precipitate out the euglobulin fraction, which is performed by adding a sufficient quantity of a saturated solution of ammonium sulfate to the serum to make a concentration of 31.5 per cent. The treated serum is allowed to stand at room temperature overnight and is then heated to 60 C. with continuous agitation to make the end product less likely to provoke serum reactions, especially in human patients. The euglobulin is removed by filtration through a special grade of filter paper and the filtrate is subjected to further processing for separation of the pseudoglobulin fraction.

It appeared desirable to determine what effect this preliminary treatment may have on virus contained in serum and, therefore, the following described experiments were carried out:

Use of Ammonium Sulfate Alone and in Combination With Heat.—The serum used in this experiment consisted of pooled serum collected from 5 horses experimentally infected with infectious anemia. The serum was divided into 3 lots. Lot 1 was untreated and held at 5 C. until the other 2 lots were tested for infectivity, a period of fifty-eight days. Lot 2, after being held at 5 C. for forty-five days, was treated with 31.5 per cent of ammonium sulfate to precipitate out the euglobulin as described previously and the precipitated serum was held under refrigeration for thirteen days before being tested for virulence. Lot 3, after being held at 5 C. for forty-seven days, was treated with 31.5 per cent ammonium sulfate in the same manner as lot 2 but, in addition, was heated to 60 C. within an hour and was held under refrigeration for eleven days before being tested for virulence.

The 3 lots were each inoculated into a normal horse. The horses inoculated with

lots 1 and 2 developed infectious anemia, whereas the horse inoculated with lot 3 failed to develop the disease. The susceptibility of this horse was confirmed by subsequent exposure to a known virus.

The results of this experiment indicate that treatment of virulent serum with 31.5 per cent of ammonium sulfate for thirteen days failed to destroy the virus, whereas treating with 31.5 per cent ammonium sulfate, heating to 60 C. within an hour and holding for eleven days, inactivated the virus.

It, therefore, appears that pseudoglobulin, when obtained from antiserums by the last mentioned method, is free from living virus.

Complete Fractionation and Dialysis.—The virulent blood serum used in this experiment was obtained from an experimentally induced acute case of infectious anemia and was divided into 3 lots.

Lot 1 was untreated and stored in the refrigerator for future use as a control sample.

Lot 2 was subjected to dialyzation to determine whether the virus would diffuse through parchment paper during dialysis. This was accomplished by placing about 50 cc. of serum in a parchment paper dialyzing bag, previously tested against leakage. The bag containing the serum was suspended in a large container of sterile, distilled water for eighteen days at 5 C. and about 65 cc. of the distilled water surrounding the bag (the diffusate) in the beaker was collected for testing for virulence.

Lot 3 was completely fractioned, the euglobulin, pseudoglobulin, and albumin fractions being separated by the usual salting-out method, employing 1/3, 1/2, and full saturation of ammonium sulfate. The final filtrate from the full saturation, after the 3 fractions had been removed, was also retained for testing.

Each precipitate was further purified by redissolving and by reprecipitation by appropriate saturation with ammonium sulfate. The serum fractions, euglobulin, pseudoglobulin, and albumin and the final filtrate were then dialyzed against running water from two and one-half to four and one-half days to remove the ammonium sulfate. This was accomplished by placing the material in small bags formed of vegetable parchment paper, which had been carefully tested for leakage before being used, and immersing in a water bath of cold, fresh, running water. The vegetable parchment used in these experiments was of the same type as that used by biological concerns as a dialyzing membrane.

Lots 1 and 2 and the euglobulin, pseudoglobulin, albumin, and final filtrate from lot 3, after being dialyzed against running water, were each inoculated subcutaneously into a normal horse. The horses inoculated with lots 1 and 2 developed symptoms of acute infectious anemia, whereas those horses inoculated with the 3 serum fractions and final filtrate from lot 3 remained normal for ten months. Pooled blood samples collected from the 4 horses inoculated with the serum fractions and final filtrate, when inoculated into a normal, susceptible horse, failed to produce infectious anemia.

This experiment indicates that the virus in virulent serum diffused through a vegetable parchment paper membrane during eighteen days' dialysis against a given amount of distilled water and that the virus was not present in the euglobulin, pseudoglobulin, or albumin fractions or in the final filtrate following dialysis in parchment paper bags against cold running water.

It appeared, therefore, that the virus was either destroyed by the action of ammonium sulfate or that it had been separated from the serum fractions by the process of dialysis. However, since the contact of 31.5 per cent ammonium sulfate for thirteen days with virulent serum had failed to destroy the virus, it seems more likely that the virus in the serum fractions was removed by dialysis.

Dialysis With Cellophane Dialyzing Membrane.—Since cellophane (cellulose film) is now being used extensively for dialysis and ultrafiltration and has been adopted for use as a dialyzing membrane by a number of biological houses in lieu of parchment paper, the following tests were carried out

to determine what effect dialysis had on virulent plasma when cellophane was used as a dialyzing membrane. Plain, transparent cellophane, Gauge 300, which has the largest porosity of the 3 grades manufactured for this purpose, was used in fabricating the dialyzing bags used in these tests.

Virulent blood serum, collected aseptically from a subacute case, was divided into 3 samples. Sample 1 was not subjected to dialysis but was held as a control sample for checking the virulence of the virus. Sample 2 was placed in a cellophane dialyzing bag which was immersed in a water bath and dialyzed for six days against cold, running tap water. Sample 3 was likewise placed in a cellophane dialyzing bag which was suspended in a beaker containing 2,000 cc. of sterile, distilled water and held in the refrigerator for sixteen days at 5 C.

Twenty-five cc. of serum from sample 1 and 50 cc. from sample 2 were each inoculated subcutaneously into a normal horse. The horse injected with sample 1 developed symptoms of acute infectious anemia in nine days whereas the horse injected with sample 2 failed to develop infectious anemia in eighty-one days but, when subsequently injected with virulent blood from the original source, developed symptoms of infectious anemia in eleven days.

Fifty cc. of sample 3 was removed from the dialyzing bag after sixteen days' dialyzation against 2,000 cc. of stationary distilled water and injected subcutaneously into a normal horse which developed symptoms of infectious anemia after thirty-three days. In this instance, virulence of the virus appeared to be somewhat modified since the incubation period was thirty-three days and the disease ran a chronic course whereas, in the horse injected with the undialyzed serum sample, the incubation period was nine days and the disease ran an acute course.

Eight hundred cc. of the diffusate (dialysate) from sample 3 was injected subcutaneously into a normal horse. Aside from a mild temperature of 101.4 F. on the fifth day, no further reaction occurred for eighty-one days. Although the mild, questionable reaction in this horse may have been caused by the presence of virus in high dilution in the diffusate, this animal when injected subcutaneously with 25 cc. of virulent blood from the original source horse, was fully susceptible, developing symptoms of the disease in fourteen days.

The results of this experiment indicate that virulent horse serum was rendered noninfectious by being placed in a cellophane dialyzing bag and dialyzed against cold, running water for six days but that when dialyzed for sixteen days at 5 C. against a given quantity of sterile, distilled water, unchanged and not in motion, the serum retained its virulence though in an apparently modified form. In a similar experiment previously described, in which vegetable parchment paper instead of cellophane was used as a dialyzing membrane, the diffusate was found to be fully virulent. A number of factors influence the process of dialysis and what effect, if any, the use of a given amount of distilled water instead of running tap water and the difference between the porosity of parchment paper and that of cellophane may have had on the results is not known. In this connection, McBain and Stuewer[19] have shown that for ultrafiltration experiments, special treatment is necessary to increase the permeability of commercial cellophane because of its small porosity.

SUMMARY AND CONCLUSIONS

1) The literature on the nature and properties of the virus of equine infectious anemia and its resistance to chemical and physical agents is briefly reviewed.

2) The virus contained in the serum from horses affected with infectious anemia, when held at 5 C., was inactivated by the action of 0.5 per cent phenol or 0.5 per cent phenol together with 0.5 per cent ether for thirty days, by 0.1 or 0.2 per cent formalin for thirty-three days, by 0.1 per cent crystal violet together with 0.05 per cent phenol for thirty days, and by phenyl mercury acetate 1:10,000 together with phenol 1:400 for thirty days.

3) The virus in horse serum held at 5 C. was not inactivated by the action of 31.5 per cent ammonium sulfate for thirteen days, nor by merthiolate 1:10,000 together with phenol 1:400 for thirty days, nor by 0.25 per cent phenol together with 0.25 per cent ether for sixty days, nor by 0.35 per cent phenol together with 0.35 per cent ether for sixty days.

4) The virus in horse brain, held at 5 C. and subjected to 0.4 per cent formalin for thirteen days or to 1.0 per cent chloroform for sixty days, was not inactivated but was apparently modified so as to increase the incubation period. The virus in horse brain was inactivated by the action of 0.4 per cent formalin for thirty to thirty-two days at 5 C. and by 1.0 per cent chloroform for ninety days at 5 C.

5) The virus in serum obtained from a carrier and heated in a water bath, the temperature of which was controlled by hand thermometers with no constant agitation of the serum, was inactivated in one test by heating at 58 C. for thirty minutes. In a second test in which pooled serum from acute cases was used, the virus was not inactivated by heating at 58 C. or 60 C. for thirty minutes. Heating at 60 C. for sixty minutes inactivated the virus but, although heating at 58 C. for sixty minutes greatly modified the virus, this treatment did not render it inactive. In a third test, serum from a carrier was heated in a pasteurizer equipped with automatic temperature control and circulating devices to assure constant agitation of the serum. In this instance, the virus was inactivated in sixty minutes by heating at 57 to 58 C., 58 to 59 C., or 59 to 60 C.

6) The virus contained in horse blood serum and brain tissue remained fully virulent after storage for at least three months at 5 C. The virus in defibrinated blood containing 20.0 per cent buffered glycerin solution retained its activity for fifteen months at 5 C., but was inactive after seven years. The virus contained in frozen horse spleen stored at temperatures ranging from —7 to —12 C. retained its virulence for thirteen and one-half months but was found to be inactive when tested after twenty-two months eight days' storage.

7) The virus in spleen and lymph gland tissue, after being dried to the consistency of a fine powder in a vacuum desiccator over calcium chloride, was fully active after storage at 5 C. for nine months ten days but the same material, after storage at 5 C. in a tightly corked vial for five years one month, was no longer active. Dehydration under high vacuum while in a frozen state (lyophilization) did not affect the stability of the virus in whole blood. Lyophilized virulent whole blood in vacuo in sealed glass ampules was apparently fully active after storage for sixteen months at room temperature and in the light.

8) The diffusate from virulent serum dialyzed in a vegetable parchment paper bag against a definite quantity of stationary distilled water was virulent while the diffusate from virulent serum dialyzed in a No. 300 cellophane bag against distilled water under similar conditions was avirulent. Virulent serum, dialyzed in a No. 300 cellophane bag against cold running water, was not infective. This indicated that the virus had been removed from the serum by dialysis against running tap water. The euglobulin, pseudoglobulin, and albumin fractions and the final filtrate from virulent serum following dialysis in parchment paper bags against running cold water contained no active virus.

9) Serum or tissue suspensions (from horses affected with infectious anemia), in which the virus had been inactivated by chemicals, heat, or other means, when injected into normal horses in large amounts, failed to protect such horses against subsequent exposure to the active virus. Similar material in which the virus was attenuated or modified by chemicals or heat when injected into normal horses produced infectious anemia. On the basis of the experimental results obtained in these studies, it is concluded that:

a. The virus shows considerable resistance against chemical disinfectants and other external influences such as heat, freezing, and desiccation.

b. It is dialyzable.

c. It is little affected by age, slowly losing its activity when stored in the refrigerator at 5 C.

d. Freezing, drying and lyophilization are effective methods for preserving the stability of the virus.

e. Formalin has fairly strong virucidal action, phenol is moderate in this respect, and chloroform is weak.

f. The virus contained in equine antiserums can be inactivated by holding it at a temperature of at least 58 to 59 C. for one hour, by certain chemical preservatives in proper concentration, or by the combined processes of fractionation, heating, and dialysis used in refining certain antiserums.

g. Tissues from horses affected with infectious anemia inactivated or attenuated by treatment with chemicals, heat, or other means have no value as immunizing agents.

References

[1]Kral, F., Macek, K., and Sobra, K.: Equine Infectious Anemia (trans.). Klin. spisy. Sk. Zverolek Brno, *11*, (1934): 25-218.

[2]Balozet, L.: Filtrabilité du virus de l'anémie infectieuse des équidés. Essai de détermination de la dimension des particules du virus. Compt. rend. Paris Académie d. Sci., *209*, (Nov. 6, 1939): 703-704.

[3]Von Mócsy, J.: Untersuchungen über die Natur des Anämievirus der Einhufer. Arch. für Wissensch. und Practische Tierheilk., *73*, (1938): 25-31.

[4]Heath, L. M.: Research on Swamp Fever or Infectious Equine Anemia, Appendix 4, Path. Div., Report of the Veterinary Director General for the year ending March 31, 1931, Dept. Agric., Canada.

[5]Luhrs: Beiträge zur Frage des Pferdewechselfiebers, Ztschr. f. Veterinärk., *33*, (1921): 66.

[6]Japanese Commission (Horse Administration Bureau, Tokyo, 1914) Report on the Results Obtained by the Special Committee for Investigation of Infectious Anemia of the Horse. Vet. J., (Dec. 1914): 604-627.

[7]Balles, Versuche über Abtötung des Virus der inf. Anämie des Pferdes durch 2% Carbolsäurelösung (Diss. Hannover 1925) Deutsche Tierarzt. Wchnschr. Jhrg., *34*, (1926): 823.

[8]Heiermann, G.: Untersuchungen über die Abtötung des Virus der infektiösen Anämie des Pferdes im Serum durch Karbolsäure und Chloroform. Dissertation, Hannover (Technische Hochschule), 1923.

[9]Fröhner and Bierbaum: Über der Resistenz des Virus der ansteck. Anämie der Pferde gegen Karbolsäure. Berl. Tierarzt. Wchnschr., *22*, (1923): 243.

[10]Semenov, M. A., and Oldenborger, N. A.: Obezzarazhivaiushchie svoistva 0.5% fenola pri konservirovanii syvorotki loshadei, soderzhashchei virus infektsionnoi anemii, Moscow, Vsesoiuznogo instituta eksperimental'noi veterinarii, Trudy (Institut de médecine vétérinaire expérimentale de l'URSS. Travaux.) *12*, (1936): 50-54. Abstract in *Veterinary Bulletin*, *8*, (1938): 27. Action of Phenol on Virus of Equine Infectious Anemia.

[11]Balozet, L.: Études Expérimentales sur L'anémie Infectieuse des Équidés, Arch. Institut Pasteur of Tunis, *24*, (1935): 297, *25*, (1936): 272-283.

[12]Von Mócsy, J.: Immunisierung und Heilversuche bei der ansteckenden Blutarmut der Pferde, Deutsche Tierarzt Wchnschr. Jhrg., *43*, (1935): 211.

[13]Balozet, L.: Action de la bile sur le virus de l'anémie infectieuse des équidés, C. R. Soc. de Biol. *99*, (1935): 818.

[14]Troitskit, I. A., and Klimov, N. M.: The Part Played by the Gastro-Intestinal Tract in the Spread of Equine Infectious Anemia (trans.), Sovyet Vet., No. 6, (1940): 15-18.

[15]Martin, L. A.: L'anémie infectieuse des équidés, IV, Adsorption du virus, Tentatives d'atténtuation du virus adsorbé. Arch. de Institut Pasteur du Maroc, II, *2*, (1940): 305, 321.

[16]Hempel, J.: Beiträge zur Kenntnis der ansteckenden Anämie der Pferde. Zschr. f. Infektions Krankheiten, parasitäre Krankheiten und Hygiene der Haustiere, *5*, (1909): 381.

[17]Carré and Vallée, H.: Recherches cliniques et expérimentales sur l'anémie pernicieuse du cheval (typho-anémie infectieuse). Rev. gén. de méd. vét., *8*, (Dec. 1906): 593-608, *9*, (Feb. 1907): 113-124.

[18]Van Es, L., Harris, E. D., and Schalk, A. F.: Swamp Fever in Horses, Bull. 94, North Dakota Agric. Exper. Sta., 1911.

[19]McBain, James W., and Stuewer, R. F.: Ultrafiltration Through Cellophane of Porosity Adjusted Between Colloidal and Molecular Dimensions. J. Phys. Chem. *40*, (1936): 1157-1168.

Experimental Infections with Eimeria Bovis in Calves

DATUS M. HAMMOND, Ph.D., LEONARD REID DAVIS, Ph.D.
and GEORGE W. BOWMAN
Auburn, Alabama

LITTLE information is available on the pathogenicity and course of infection of the various species of coccidia in cattle. Such knowledge has been difficult to obtain because most infections occurring naturally involve two or more species. Information concerning the course of infection of the most pathogenic species is necessary as a foundation for developing methods of prevention and control of coccidiosis. *Eimeria zurnii and Eimeria bovis* are known to be the two species most frequently involved in clinical coccidiosis. The purpose of this paper is to present data on the symptoms, course of infection, and relationship between the number of oöcysts ingested and the severity of the disease in calves experimentally infected with *E. bovis* (synonym *Eimeria smithi*).

MATERIALS AND METHODS

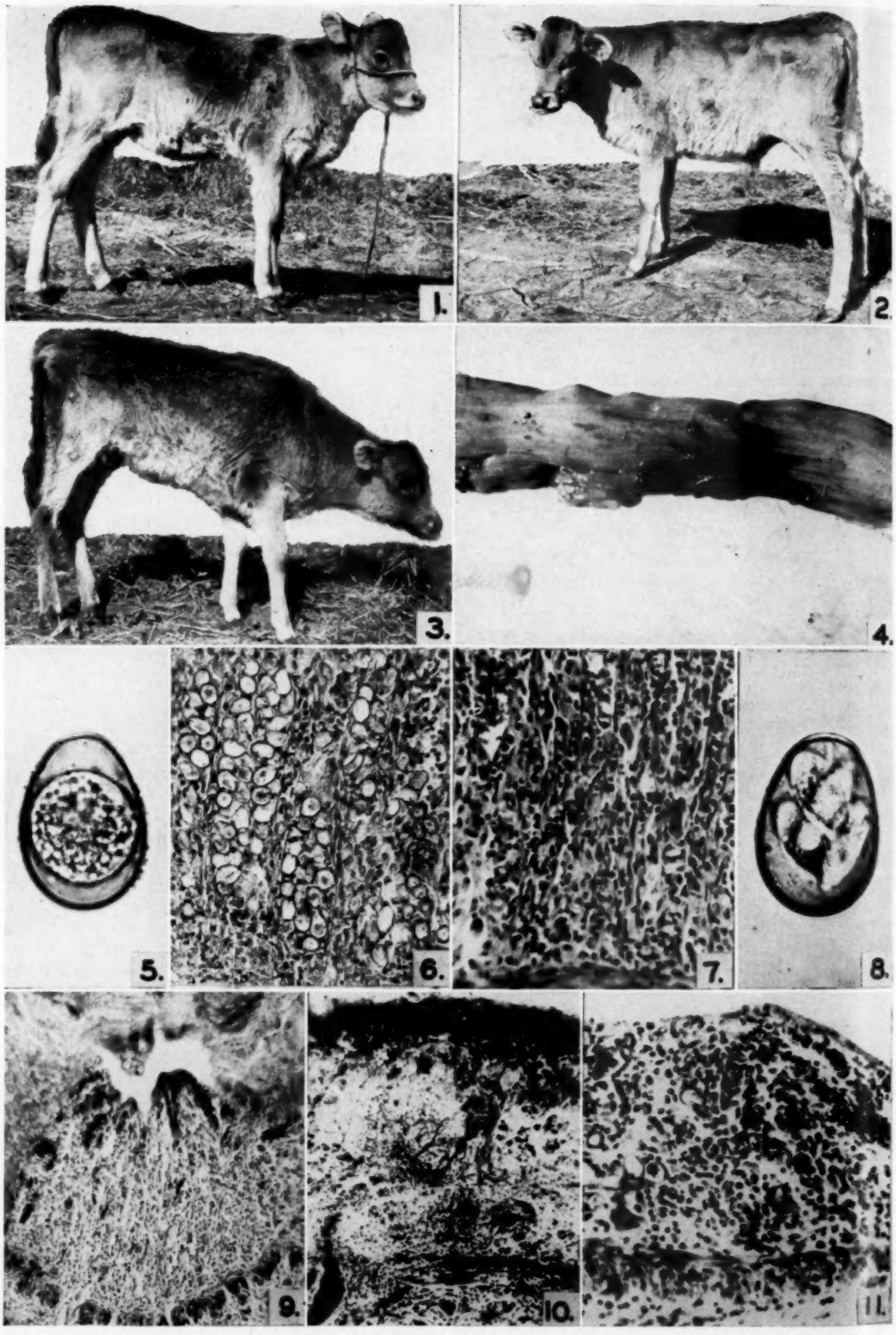
Twelve of the 15 calves used in these experiments were grade Jerseys, 2 (519 and 497) were grade Herefords, and 1 (496) was a grade Holstein-Friesian. All except 2 (518 and 519) were males. The calves were obtained when they were 1 day old or less and were kept under conditions designed to minimize the opportunities for natural infection with coccidia. Each calf was placed in a separate clean pen, and in the earlier experiments, a special muzzle was placed on the calf as an additional precaution against infection. It was found that the method of isolation was effective in preventing natural infection, especially where the calves were kept in movable outside pens on ground not previously used by cattle. Consequently, in the later experiments, the calves were not muzzled. Feeding of a mixture of grain and chopped hay was begun when the calves were about 1 month old. At the time the experiment was begun, all calves, except 2, were in a good state of nutrition and showed no evidence of disease. Calves 488 and 503, both of which later developed pneumonia, appeared unthrifty at the beginning of the experiment. None of the calves discussed in this paper showed oöcysts of *E. bovis* prior to the experimental infection, but 5 (517, 519, 496, 493, and 509) had light infections with other species of coccidia.

In the inoculum used for producing infections, no species of coccidia other than *E. bovis* was seen. The material used in experiments 1 to 4 was obtained from fecal specimens of calves having natural infections with *E. bovis*, while the material used in experiments 5 and 6 came from experimentally infected calves. With the exception of experiment 5, infectious material from the same source was used for all calves in any one experiment. In experiment 5, two different mixtures of fecal specimens were used, but these two mixtures came from calves which had been inoculated at the same time with portions of a single mixture of fecal specimens.

Sporulation of the oöcysts in the material was brought about by a method already described (Hammond and Davis[1]). The appearance of oöcysts before and after sporulation is shown in plate 1, fig. 5 and 8. The concentration of oöcysts in the material was estimated with the aid of a hemacytometer, except for 3 cases (calves 526, 483, and 517), in which the oöcysts were isolated and counted with the aid of a micromanipulator. In each case, the fecal material containing oöcysts was washed thoroughly to remove the potassium dichromate solution, placed in a flask, diluted with water, warmed, and fed through a rubber nipple just prior to the afternoon feeding of milk.

Daily fecal samples were obtained from each calf beginning about ten days after birth and continuing through the period covered by the experiments. The routine of preparation and examination of samples was similar to that described by Boughton.[2] This involved straining a tablespoonful of sample through a 60-mesh sieve with a liter of water, allowing it to sediment over night, pouring off all but 150 cc., removing a 15 cc. sample, centrifuging, pouring off water, and adding sugar solution, centrifuging again, removing a portion from the surface with a wire loop, 5 mm. in diameter, and examining it as a hanging drop. The slide was examined with 10x oculars and 4

From the U. S. Regional Animal Disease Research Laboratory, Bureau of Animal Industry, Agricultural Research Administration, U. S. Department of Agriculture, Auburn, Ala.



—*Photography by Leonard Reid Davis*

mm. objective. If few oöcysts were present, the entire drop was examined; if moderate numbers were present, an area one field wide across the greatest diameter of the drop was examined, and the number of oöcysts multiplied by 20 (the average drop was approximately 20 fields wide); if large numbers were present, one field was examined, and the number of oöcysts multiplied by 200 (the average drop included about 200 fields). Thus, the result of this examination represented the estimated number of oöcysts in a loopful from a flotation of one-tenth tablespoonful of fecal sample. It is obvious that this method gave only a rough approximation of the numbers of oöcysts present, but since the same method was used for all samples an idea as to comparative numbers was obtained.

The experiments covered a period of approximately six months. Experiment 1 was begun on Aug. 16, 1943, experiment 2 on September 24, experiment 3 on October 8, experiment 4 on October 22, experiment 5 on December 1, and experiment 6 on Jan. 4, 1944. In experiments 3 and 6, the inoculum was given at the same time to each of the calves on the same experiment. In experiment 4, calf 497 was inoculated on October 22, 502 on November 4, and 503 on November 9. In experiment 5, calf 517 was inoculated on December 1, calf 519 on December 4, calf 518 on December 6, and calf 526 on Jan. 4, 1944.

In each experiment, abnormalities in appearance and behavior were recorded. In all but 3 of the calves (526, 483, 488), a daily record of temperature, taken at approximately the same time each day, was kept prior to and during the period of oöcyst discharge. No form of treatment was given to any of the calves to alleviate the symptoms of coccidiosis, and autopsies were held as soon as possible on the calves which died to determine the nature and extent of pathologic changes, and the location, species, and relative numbers of coccidia.

RESULTS

The results of experiments 1 to 5 are presented in table 1 and the results of experiment 6 in figure 1. All calves except one became infected with *E. bovis* following inoculation with oöcysts. Calf 488 showed no evidence of infection at the time it died of pneumonia twenty-two days after receiving an inoculum containing about 125,000 oöcysts. One other calf (503) apparently had only a very light *E. bovis* infection following inoculation of the same number of oöcysts, and this calf had pneumonia and an abscessed liver when it was killed in a moribund condition twenty-eight days after receiving the inoculum.

The 5 calves (526, 483, 517, 518, and 519), each of which received from 1,000 to 10,000 oöcysts when it was 1 to 11 days old, showed only slight or no symptoms during the infection.

Calf (510), which received 25,000 oöcysts when it was 2 months old, was diarrheic on the twentieth, twenty-fifth, and twenty-sixth days following inoculation. The diarrhea on the last two days was of a different

Explanation of Plate on Opposite Page

All sections stained with iron alum-haematoxylin.

Figs. 1, 2, 3—Calves 510, 508, and 509 on the twenty-fifth day after receiving about 25,000, 125,000 and 500,000 oöcysts of Eimeria bovis, respectively.

Fig. 4—Middle colon of calf 509 showing bloody membrane (about ½ natural size).

Fig. 5—Freshly discharged oöcyst of Eimeria bovis. x 1000.

Fig. 6—Section of cecum of calf killed eighteen days after receiving about one million oöcysts of Eimeria bovis, showing lower portion of mucosa with Eimeria bovis oöcysts in intestinal glands. x 140. Fixed in Zenker's.

Fig. 7—Section of cecum of calf 509, showing obliteration of intestinal glands, and scattered Eimeria bovis oöcysts in lower portion of mucosa. x 140. Fixed in Zenker's.

Fig. 8—Sporulated oöcyst of Eimeria bovis. x 1000.

Fig. 9—Section of middle colon of calf 502 showing island of intact mucosa and submucosa surrounded by noncellular membrane. Glands in mucosa obliterated. x 80. Fixed in Helly's.

Fig. 10—Section of upper middle colon of calf 502 showing replacement of mucosa by noncellular membrane, submucosa intact. x 80. Fixed in Helly's.

Fig. 11—Section of upper colon of calf 502 showing congestion, destruction of glands, and scattered oöcysts of Eimeria bovis. x 140. Fixed in Zenker's.

character from that of the first day, and probably was only indirectly or not at all associated with the *E. bovis* infection. The calf showed no evidence of depression (plate 1, fig. 1).

Excluding the 2 calves previously discussed (488 and 503), which developed pneumonia, all the calves which received 125,000 oöcysts or more showed marked symptoms during the infection. Five out of 7 of these calves died or were killed in a moribund condition. Each of the 2 calves (495 and 508) which survived had received 125,000 oöcysts, while each of those which died had received 125,000 to 1 million oöcysts.

The course of symptoms of a typical case (509) was as follows: During the seventeen days following the inoculation, no abnormalities in appearance or behavior were noted. In the morning of the eighteenth day, the feces were diarrheic and dark in color, and in the afternoon the feces appeared streaked with blood. On the nineteenth day, the diarrhea became more severe, and was characterized by frequent, spurting discharge of watery, bloody fluid and clots of blood and feces. Beginning on this day, the calf ate no more solid food. On the twentieth day, the diarrhea continued as on the previous day, and, in addition, tubular pieces of bloody tissue appeared. Most of these pieces were several inches long with a diameter slightly less than that of the colon. Beginning on this day, the calf showed depression. On the

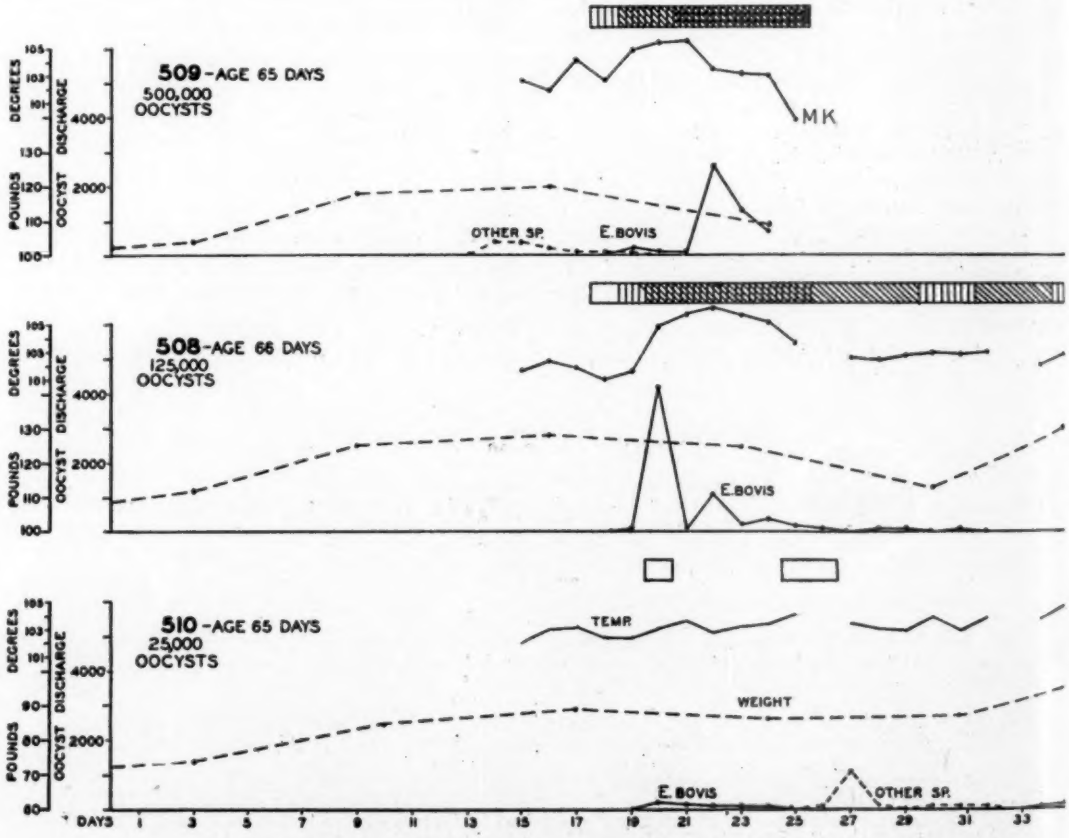


Chart 1—Results of experiment 6, showing oöcyst discharge, weight, temperature, and abnormalities in fecal discharge of calves 510, 508, and 509 following inoculation of oöcysts of Eimeria bovis in numbers of approximately 25,000, 125,000, and 500,000, respectively. Abnormalities in fecal discharge shown in the rectangles: blank = diarrhea, vertical lines = bloody diarrhea, diagonal lines = tissue discharged, two horizontal lines inside rectangle = tenesmus. MK = moribund, killed.

twenty-first day, the quantity of material discharged was reduced, and slight tenesmus was evident. On the twenty-second day, tenesmus was more frequent and pronounced, and only a small amount of material was discharged. This consisted of bloody fluid, bits of tissue, and tan coagulated material. The hindquarters were badly soiled with the bloody discharge; the eyes of the calf began to sink in; only a portion of the milk was taken on this day and no milk was taken afterward; dehydration and weakness were pronounced. These symptoms progressed until the twenty-fifth day (plate 1, fig. 3), at which time it was evident that the calf would die within twenty-four hours; therefore it was killed. At autopsy, all the viscera except the lower ileum, cecum, and colon appeared normal. The posterior three feet of the ileum, the cecum, and the colon were filled with semifluid, bloody material. The cecum and colon were swollen and thickened, and showed evidence of recent hemorrhage, especially in the upper colon. A bloody membrane, which was easily separable from the underlying mucosa, was present in parts of the colon and cecum (plate 1, fig. 4). Numerous oöcysts of *E. bovis* were seen in scrapings from the cecum and colon. Sections of the cecum and colon showed congestion, destruction of the intestinal glands, and scattered oöcysts of *E. bovis* (plate 1, fig. 7).

The symptoms were similar in the other calves with severe *E. bovis* coccidiosis. The diarrhea began on the eighteenth day in all these calves except 502, in which it began on the twentieth day. In 3 of the calves (502, 497, 496), tenesmus appeared relatively later than in the other 2 calves (509, 493). Four of the calves with marked symptoms of coccidiosis died or were killed because of their moribund condition by the twenty-fifth day, while the fifth (496) survived to the twenty-seventh day. At autopsy, none of these calves showed evidence of any significant degree of pneumonia. The colon, cecum, and, in some cases, the lower ileum were filled with sloughed tissue and bloody fluid. The small intestine showed congestion in varying degree, but there was no evidence of marked gross pathologic change. The cecum and colon, on the other hand, were thickened, and showed severe hemorrhage and erosion. In 4 of the calves, the greatest damage occurred in the lower half of the colon. In 3 cases, the upper colon and cecum showed fresh hemorrhage, petechial and diffuse, while the lower colon was dark in color. A tan, yellow or greenish-yellow membrane was present in patches or in a continuous layer over the surface of the cecum and colon. In one of the calves (502), a gelatinous edema occurred on the outer surface of the colon. Oöcysts of *E. bovis* were found in large numbers in the cecum and colon. In 2 cases, oöcysts were also seen in the eight to twelve inches of the ileum adjacent to the ileocecal valve, and in 1 case, oöcysts were seen in the ileum as far as six feet above the valve. Oöcysts of other species of coccidia were not found in any of these calves. Relatively small numbers of the macroscopic schizonts described by Boughton[3] as asexual stages of coccidia, probably *E. bovis*, were seen in the lower third of the small intestine.

In sections of the cecum and colon, oöcysts of *E. bovis* were seen (plate 1, fig. 6). The major pathologic changes consisted of congestion, extensive destruction of the intestinal glands (plate 1, fig. 11), formation of a membrane (plate 1, fig. 9), and, in some areas, denudation of the mucosa (plate 1, fig. 10). In one case (502), sections from the middle and lower colon showed replacement of the entire mucosa and submucosa by a noncellular membrane, except for small areas where the mucosa and submucosa were still present (plate 1, fig. 9).

It is apparent that the sexual phase of the life cycle of *E. bovis* was the one directly concerned in causing damage to the host, as evidenced by (a) the correlation in time of first appearance of symptoms and the beginning of oöcyst discharge and (b) the localization of severe pathologic changes in the cecum and colon, where the sexual stage occurs.

Of the 2 calves which survived severe coccidiosis, 1 (508) showed symptoms of more acute illness than the other (495). Calf 508 had severe bloody diarrhea from

the nineteenth to twenty-fourth day following inoculation; ate no grain mixture from the twenty-first to twenty-fifth day; took only a small amount of milk on the twenty-third day; and showed depression, including sunken eyes, from the twenty-second to the twenty-fourth day (plate 1, fig. 2). The feces remained soft until the thirty-seventh day, and strips of tissue or blood or both appeared in the feces until the forty-fourth day. There was marked loss of weight during the severe diarrhea and a rapid gain following recovery from the acute stage of the infection (fig. 1). Calf 495 did not have typical bloody diarrhea and showed no depression. However, strips of bloody tissue appeared in the feces from the twentieth to thirty-third day, and an intermittent diarrhea persisted until the eighty-fifth day. In two other cases, not included in this report, persistent diarrhea followed recovery from severe *E. bovis* coccidiosis.

All the calves which had marked symptoms of coccidiosis showed a definite rise in temperature two to three days after the first appearance of diarrhea. The daily temperatures of calves 508 and 509 are shown in figure 1, while those of the remaining 5 calves are shown in figure 2. The peaks of temperature in the seven calves ranged from 104.4 to 106.6 F. The abnormally high temperature lasted only from one to three days in most cases, then returned toward normal. However, in one calf (508), the temperature was 104.8 F. or above for five days. In the four calves (517, 518, 519, 510) with slight or no symptoms during infection, no abnormalities of temperature occurred during the infection.

The discharge of oöcysts began in 1 case (502) on the sixteenth day following the inoculation, in 3 cases (519, 497, 496) on the eighteenth, in 4 cases (495, 493, 508, 509) on the nineteenth, in 4 cases (517, 518, 483, 510) on the twentieth, and in 2 cases (526, 503) on the twenty-first. Six of the 7 calves which developed marked symp-

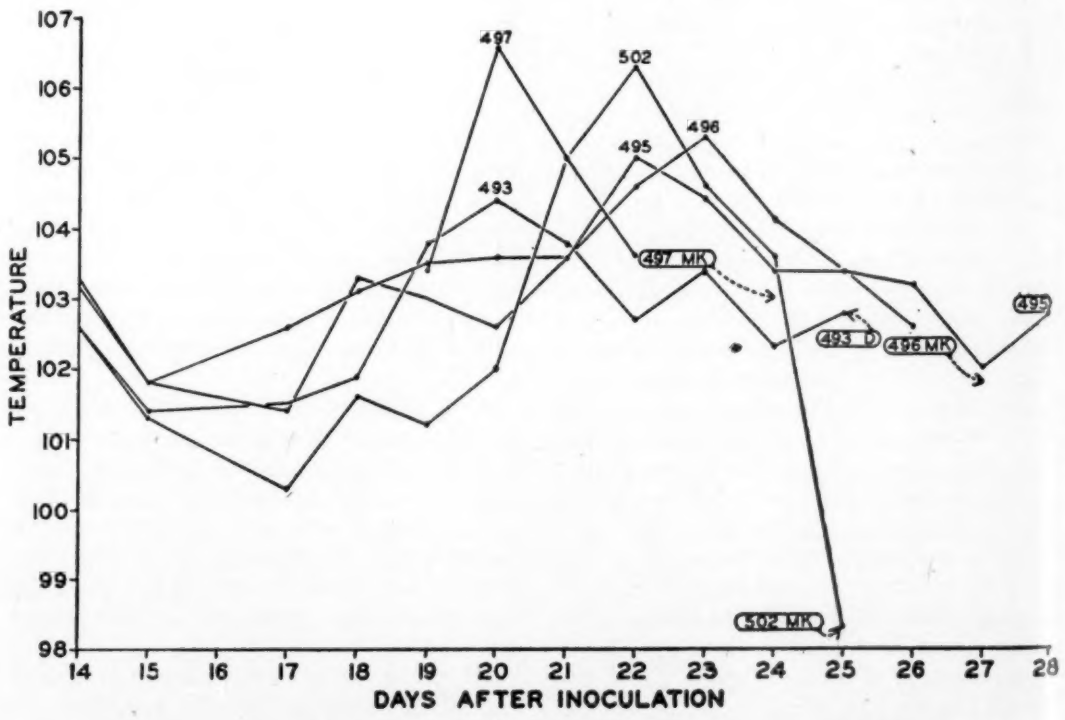


Chart 2—Temperatures of five calves which showed marked symptoms of cociddiosis following inoculation of Eimeria bovis oöcysts. MK = moribund, killed; D = died.

TABLE I—Experimental Infection with Eimeria Bovis in Calves Not Previously Infected with Eimeria Bovis

Calf No.	Age (days)	Experiment number	Thousands of oöcysts given	Abnormalities in Fecal Discharge: Type	Abnormalities in Fecal Discharge: Time (days after inoculation)	Discharge of Oöcysts: Peak (thousands) and day after inoculation	Discharge of Oöcysts: Time (days after inoculation)	Remarks
526*	1	5	1.0†	Slight diarrhea Bloody feces	22-24 23	0.8 (22)	21-44	No perceptible after-effects.
483*	10	1	1.2†	None		1.6 (35)	20-38‡	No perceptible after-effects.
517	10	5	2.6†	None		2.3 (22)	20-32‡	No perceptible after-effects.
518*	11	5	5.0	None		1.1 (22)	20-29‡	No perceptible after-effects.
519	10	5	10.0	Diarrhea	20, 22-23	1.1 (21)	18-31‡	No perceptible after-effects.
488*	29	2	125.0	None				Symptoms of pneumonia early in experiment. Died on 22nd day. Lungs consolidated, no coccidia found in intestine.
503*	27	4	125.0	Bloody diarrhea	25	0.05 (22)	21-24	Symptoms of pneumonia prior to beginning of experiment. Moribund, killed on 28th day. Lungs consolidated, liver enlarged, abscessed.
502*	28	4	125.0	Diarrhea Bloody diarrhea Tenesmus	20 21-25 24-25	6.4 (22)	16-25	Moribund, killed on 25th day. Cecum and colon showed much pathologic change, oöcysts of *E. bovis*.
495*	26	3	125.0	Diarrhea Diarrhea + tissue	18-20 21-33	5.6 (21)	19-47	Intermittent diarrhea to 85th day.
497*	39	4	250.0	Diarrhea Bloody diarrhea Tenesmus	18 19-24 24	0.2 (21)	18-24	Moribund, killed on 24th day. Cecum and colon showed much pathologic change, oöcysts of *E. bovis*.
496	26	3	500.0	Bloody diarrhea Tenesmus	18-27 24-27	3.4 (20)	18-27	Moribund; killed on 27th day. Cecum and colon showed much pathologic change, oöcysts of *E. bovis*.
493	31	3	1000.0	Diarrhea Bloody diarrhea Tenesmus	18 19-25 20-25	1.7 (23)	19-25	Died during night of 25th day. Cecum and colon showed much pathologic change, oöcysts of *E. bovis*.

*No oöcysts of any species found in samples prior to the infection involved in this experiment.
†Oöcysts isolated and counted with the aid of a micromanipulator.
‡Another inoculum given at this time; therefore, the remaining portion of the period of discharge resulting from the original inoculation could not be determined.

toms began discharging oöcysts either the same day that diarrhea first appeared or one day later, while in 1 of the 7 calves (502), oöcysts were observed in the feces four days before diarrhea set in. The discharge of oöcysts reached its peak in concentration on the twentieth day in 3 cases (496, 510, 508), on the twenty-first day in 3 cases (519, 495, 497), on the twenty-second day in 6 cases (526, 517, 518, 503, 502, 509), on the twenty-third day in 1 (493), and on the thirty-fifth day in 1 (483). After the day on which the oöcysts were given off in greatest concentration, there was a gradual, irregular decrease in numbers. Their discharge in considerable numbers lasted no more than six or seven days in most cases. Oöcysts were then given off in small numbers for two to three weeks, after which they could no longer be found in the samples. The concentration of oöcysts discharged was higher in 2 calves (502, 495) which had received 125,000 oöcysts than in the calves which had received smaller or larger numbers. The calves (526, 483, 517, 518, 519) which had received the smallest numbers of oöcysts (1,000 to 10,000) discharged oöcysts in approximately as great a concentration as the calf (493) which had received 1,000,000 oöcysts. Lower concentrations of oöcysts were found in the fecal samples from a calf (497) which had fatal coccidiosis than in samples from any of the other calves except the 2 with pneumonia, 1 of which discharged no oöcysts, the other only relatively few. Therefore, with the methods used, it appears that in coccidiosis produced by *E. bovis*, the concentration of oöcysts discharged is not closely correlated with the severity of infection. However, in all the calves on which autopsies were held following severe coccidiosis, there were large numbers of oöcysts in the cecum and colon. Therefore, it is probable that in the calves with severe coccidiosis, the pathologic changes which occur alter what might be called the normal process of discharge of oöcysts which takes place in calves without marked symptoms. Also, it is possible that in the fecal samples from calves with coccidiosis, the presence of tissue and coagulum decreases the accuracy of oöcyst detection by the method of straining, sedimentation, and flotation.

DISCUSSION

The cases of coccidiosis in calves and older animals investigated by Bruce,[4] apparently involved several species of coccidia, including *E. bovis*. In experimental infections, he found that the incubation period was fourteen days as compared with the eighteen-day period characteristic of the infections in the present study. Also, Bruce observed no rise of temperature, and death was usually preceded by convulsions. Yakimoff, Belawine, and Rastegaieff[5] stated that coccidial colitis may be produced by mixed infection with *E. zurnii* and *E. bovis* (*smithi*), or by infection with either species singly.

Wilson and Morley[6] gave material containing oöcysts only of *E. bovis* (*smithi*) to a calf and found diarrhea and oöcysts on the ninth and tenth day, respectively, and diarrhea and oöcysts from the seventeenth to twentieth days. Oöcysts were again found on the thirty-first to thirty-eighth days. Both of the latter discharges corresponded in time with the results found in the present study, but no negative period occurring in the middle of the period of oöcyst discharge has been found in the present study. Wilson and Morley were unable to produce severe infections experimentally with any of the species of coccidia with which they worked, and they found no correlation between the size of inoculum given and the resulting infection. They suggested that some predisposing factor influenced the course of infection. In the present study, infections were produced by inoculation of as few as 1,000 oöcysts, and the effect of an infection on an animal was closely correlated with the numbers of oöcysts given. Infections caused by oöcysts in numbers of 25,000 and below were accompanied by slight or no symptoms, while infections caused by numbers of 125,000 oöcysts and above were accompanied by marked symptoms, and these infections terminated in death in 5 of 7 ani-

mals. These results indicate that under the conditions of these experiments in calves previously uninfected with *E. bovis*, the number of oöcysts ingested was the factor of greatest importance in determining the severity of infection.

SUMMARY

1) In six experiments conducted at different times, 15 calves previously uninfected with *Eimeria bovis*, varying in age from 1 day to 2 months, were inoculated with material containing from 1,000 to 1,000,000 oöcysts of *E. bovis*.

2) All the calves except 1 became infected, as evidenced by (*a*) discharge of *E. bovis* oöcysts beginning on the sixteenth to twenty-first day following inoculation and (*b*) diarrhea and/or bloody diarrhea in all calves which received larger doses and some of the calves which received smaller doses.

3) Two calves developed pneumonia and 1 of these showed no evidence of having become infected as a result of the inoculation, while the other apparently developed only a very light infection.

4) Excluding the 2 calves which developed pneumonia, all 7 calves which received 125,000 oöcysts or over developed marked symptoms beginning, in all calves except 1, on the eighteenth day following inoculation, and 5 of these died or were killed in a moribund condition twenty-four to twenty-seven days after the inoculation. A definite rise in temperature to a peak ranging from 104.4 to 106.6 F. occurred in each of the 7 calves during the time it was showing symptoms.

5) All the calves which died showed marked pathologic changes in the cecum and colon, and numerous oöcysts of *E. bovis* in these regions.

6) In most of the calves, there was a relatively heavy discharge of oöcysts for five or six days, beginning on the eighteenth to twentieth day following inoculation. Small numbers of oöcysts were found in the feces for two to three weeks after the heavier discharge ceased.

References

[1]Hammond, Datus M., and Davis, Leonard Reid: An Improved Method of Sporulating Oöcysts in Bovine Fecal Material. Am. J. Vet. Res., *5*, (1944): 70-71.

[2]Boughton, Donald C.: Sulfaguanidine Therapy in Experimental Bovine Coccidiosis. Am. J. Vet. Res., *4*, (1943): 66-72.

[3]Boughton, Donald C.: An Overlooked Macroscopic Intestinal Lesion of Value in Diagnosing Bovine Coccidiosis. North Am. Vet., *23*, (1942): 173-175.

[4]Bruce, E. A.: Bovine Coccidiosis in British Columbia, with a Description of the Parasite, *Eimeria canadensis* sp. n. J.A.V.M.A., *58*, (1921): 638-662.

[5]Yakimoff, W. L., Belawine, W. S., and Rastegaieff, E. F.: Zur Frage ueber Rindercoccidien im Nordkaukasus. Arch f. Protistenk., *70*, (1930): 643-654.

[6]Wilson, I. D., and Morley, L. C.: A Study of Bovine Coccidiosis, II. J.A.V.M.A., *82*, (1933): 826-850.

Calcium Boro-Gluconate-Saccharate: Preparation, Toxicity and Effect on Serum Calcium Level in Cows

A. APPLEBY, B.S., D.V.M., M.S.
Blacksburg, Virginia

"MILK FEVER" of cows is the popular name for the clinical syndrome evinced by lactating cows suffering from hypocalcemia. The hypocalcemic condition is brought on by the loss of calcium salts in the milk in excess of the calcium made available to the animal in its feed or by the calcium stores of the body. According to Little and Wright[1], milk fever usually occurs when the serum calcium falls to about 4.1 mg.

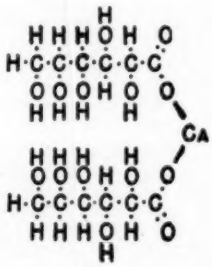


FIG. 1 A
CALCIUM GLUCONATE

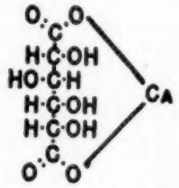


FIG. 1 B
CALCIUM SACCHARATE

per cent, the normal level, as reported by Newton[2], being around 10.0 mg. per cent.

The rational therapeutics of this condition is the administration of calcium salts easily utilizable to the animal. These are made available most rapidly through the agency of intravenous injection. The calcium salts most widely used by veterinarians at present are calcium lactate, calcium chloride, and calcium gluconate[3,4]. Carratala[5] reports that, in dogs and rabbits, when an intravenous injection rate of 1 cc. per minute is used, the comparative lethal dosages of these three salts are 1.00 : 1.17 : 1.82, respectively, while when an injection rate of 2 cc. per minute is used the ratio is 1.00 : 1.44 : 2.11.

It may be assumed that the therapeutic agent which will make available to the animal more calcium per unit volume administered will be the more efficient one. Since the clinical efficacy and comparative safety of calcium gluconate have been established by experiment and experience, a search was instituted for a compound closely related to this salt but containing more calcium per unit weight. The compound finally chosen was calcium d-saccharate. This is the calcium salt of d-saccharic acid, and a study of figures 1A and 1B shows that one unit weight of calcium d-saccharate contains 39.0 per cent more calcium than does the same weight of calcium d-gluconate.

The main difficulty encountered in preparing a stable solution of calcium d-saccharate was that of insolubility; the compound is insoluble in water. De Carli[6], by the addition of 3.67 per cent of boric acid, increased the solubility of calcium d-gluconate in water from 3.3 per cent to 39.0 per cent, and Dryerre and Greig[7] utilized boric acid in the preparation of a 20.0 per cent solution of calcium gluconate. These authors believe that a complex of the type calcium gluconate-boric acid-water is formed which accounts for the action in increasing the solubility. Pasternack and Giles[8] claim that calcium gluconate and calcium saccharate in water manifest a mutual solubility-increasing effect. The determination of the effect of boric acid on the solubility of calcium d-saccharate and the preparation of a stable solution containing boric acid, calcium d-gluconate, calcium d-saccharate and water are included in this investigation.

The preparation of this solution was followed by a study of its effect on the serum calcium level in cows. Godden and Duckworth[9], working with a 10.0 per cent solu-

---

A modification of a thesis presented in partial fulfillment of the requirements for the degree of Master of Science, Virginia Polytechnic Institute, Blacksburg, Va.

Aided by a grant from the Virginia Academy of Science.

The author is now with the Armed Forces.

tion of calcium gluconate, report that "only about 15.0 per cent of the calcium injected could be found in the serum twelve min-

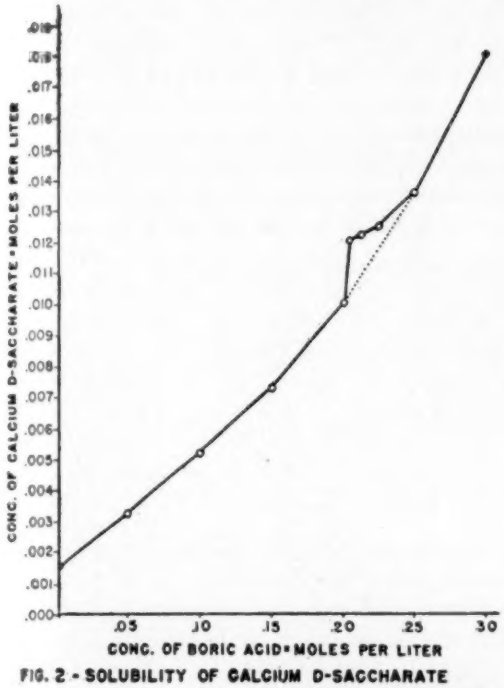


FIG. 2 - SOLUBILITY OF CALCIUM D-SACCHARATE IN BORIC ACID SOLUTION

utes after injection." Glaser[10] also states that calcium salts injected into the animal system soon disappear from the blood.

EXPERIMENTAL PROCEDURE AND RESULTS

The effect of different concentrations of boric acid on the solubility of calcium d-saccharate in water was determined as follows: An excess amount of powdered calcium d-saccharate was added to each of the following concentrations of boric acid: 0.0000 molar, 0.0500 molar, 0.1000 molar, 0.1500 molar, 0.2000 molar, 0.2092 molar, 0.2209 molar, 0.2268 molar, 0.2500 molar, and 0.3000 molar. These mixtures were allowed to stand at room temperature for twenty-four to forty-eight hours with intermittent shaking. They were then cooled to 20 C., shaken vigorously, and filtered through Whatman no. 1 filter paper. The concentration of dissolved calcium d-saccharate in each of the filtrates was determined by evaporating three 10 cc. samples of each filtrate, weighing the residue from each sample, and correcting for the weight of boric acid present. The results of these determinations are graphically represented in figure 2.

Since boric acid alone does not enhance the solubility of calcium d-saccharate sufficiently for its efficient use as a parenteral calcium solution, it was decided to utilize the combined dissolving powers of boric acid and calcium d-gluconate. The following formula was used:

Calcium d-gluconate	100.0 Gm.
Boric acid	10.0 Gm.
Calcium d-saccharate	37.5 Gm.
Distilled water	500.0 cc.

The gluconate, boric acid, and water were mixed and boiled until solution was complete. To the boiling solution the saccharate was added slowly to prevent excessive foaming. The final solution was then filtered through cotton. Stability studies show that the solution is susceptible to fungus contamination but is stable physically, samples which had been sterilized for fifteen minutes at 12 pounds steam pressure remaining free of precipitation or crystallization at the end of six months at room temperature.

The toxicity of this boric acid-saccharate-gluconate solution was compared with that of a commercial solution containing boric acid and calcium gluconate. For this comparison, a calcium boro-gluconate solution, product of a veterinary pharmaceutical concern, was chosen. The test animals were 7-month-old albino rabbits, males and females, all reared under identical conditions. Three of the rabbits (5, 10, and 18) received calcium boro-gluconate solution (C-B-G); three others (7, 8, and 12) received calcium boro-saccharate-gluconate solution; one rabbit (9) received physiologic saline solution. All solutions were injected into the external auricular vein by means of a 22-gauge needle at the rate of 2 cc. per minute, except in the case of the saline injection which was done at 2 cc. per minute

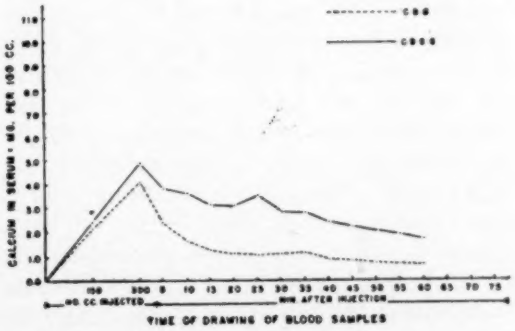


FIGURE 3 - COURSE OF SERUM CALCIUM LEVEL FOLLOWING C-B-G AND C-B-S-G INJECTIONS [AVERAGE]

until 200 cc. had been injected and then at 4 to 5 cc. per minute. The results of this section of the experiment are listed in table 1.

Since the C-B-S-G solution was found to be comparatively nontoxic for rabbits, it was felt that it would be safe to inject it into cows. An investigation was undertaken to determine whether the higher calcium content of the C-B-S-G solution (32.58 per cent higher) would be manifested in the effects of the two solutions on the serum calcium level of cows.

Three cows were used for this investigation, each animal receiving 300 cc. of the solution under test. The schedule of injections and blood withdrawals is shown in table 2. The solutions were injected slowly into the jugular vein and blood was withdrawn from the same vein into 50 cc. Erlenmeyer flasks. Calcium determinations were run in duplicate on the serum from each sample. In these determinations, the Kramer-Tisdall technique[1], utilizing 2 cc. of serum, was employed. The results of these determinations are listed in figure 3.

TABLE 1—Comparative Toxicity of C-B-G and C-B-S-G Solutions for Rabbits

RABBIT NO.	WEIGHT (GRAMS)	SEX	SOLUTION	RATE OF INJECTION	LETHAL DOSE	
					NET	AVERAGE
5	3170	M	C-B-G	2 cc./min.	98 cc.	0.031 cc./gm.
10	2558	F	C-B-G	2 cc./min.	100 cc.	0.039 cc./gm.
18*	2520	F	C-B-G	2 cc./min.	69 cc.	0.027 cc./gm.
7	3230	M	C-B-S-G	2 cc./min.	127 cc.	0.039 cc./gm.
8	2882	F	C-B-S-G	2 cc./min.	123 cc.	0.042 cc./gm.
12	2895	F	C-B-S-G	2 cc./min.	114 cc.	0.039 cc./gm.
9	3042	M	0.85% NaCl	variable†	272 cc.	0.090 cc./gm.

*Severely infested with ear-mites.
†2 cc. per minute until 200 cc. had been injected; 4-5 cc. per minute thereafter.

DISCUSSION

The determination of the solubility of calcium d-saccharate in various concentrations of boric acid shows that the solubility increases as the boric acid solution becomes more concentrated. Of especial interest is the break occuring in the solubility curve in the region between 0.20 and 0.25 molar boric acid solutions. The significance of this break asserts itself in the light of the following statement by Dryerre and Greig: "When calcium gluconate is dissolved in boric acid, we have reason to believe that a new chemical compound is formed, for which, in the absence of precise information regarding its structure, we suggest the name 'calcium borogluconate.'"

It is a common occurrence in plotting curves in solubility tests, conductance tests, freezing-point change determinations, etc., to find a break in an otherwise smooth curve at the point, or in a region, where a new compound is formed. In view of this, and in view of the very close chemical relationship of calcium d-saccharate to calcium d-gluconate, it is not untenable to assume that at the break in the saccharate-boric acid curve, a new compound, calcium boro-saccharate, is formed. Based on this assumption, the mixture prepared in the course of this investigation has been called calcium boro-saccharate-gluconate, the final solution being known as C-B-S-G solution.

The tests for the determination of the comparative toxicities of the C-B-G and C-B-S-G solutions show that the ratio of toxicity is, respectively, approximately 1.00 : 1.25. Taking the figures reported by Carratala for the 2 cc. per minute injection rate into rabbits, the comparative lethal dosages of calcium lactate, calcium chloride, calcium gluconate, and C-B-S-G solutions are 1.00 : 1.44 : 2.11 : 2.64, respectively. During the present investigation, cows receiving 300 cc. of the C-B-S-G solution intravenously showed no evidence that this solution was in any way toxic as compared with cows receiving the same amount of C-B-G solution. That the death of the rabbits receiving the C-B-G and C-B-S-G solutions was due to qualitative rather than wholly quantitative effects is shown by the fact that rabbit 9 was able to survive the injection of a quantity of physiologic saline solution more than twice that of either of the other solutions.

The previously mentioned statements to the effect that intravenously injected calcium disappears rapidly from the blood stream of cows have been corroborated in this investigation. With both solutions, the serum calcium level rises rapidly during injection and falls rapidly following in-

jection, but it rises higher, falls more slowly, and does not fall as far after injection of the C-B-S-G solution as it does following the injection of C-B-G solution. Following the use of C-B-S-G solution, there is a secondary peak in the serum calcium level about one-half hour after injection. This phenomenon is of importance since it may be a starting point in the determination of the actual fate of injected calcium salts in the animal body.

TABLE 2—Variation in Serum Calcium Level Following Injections of C-B-G and C-B-S-G Solutions

Trial No.	Cow	Solution Injected	Time Bled	Sample No.	Mg. Ca per 100 cc. Serum
1	Veeman Belle	C-B-S-G	B.I.	1	11.3*
			150 cc. I.C.	2	14.5
			300 cc. I.C.	3	15.0
			3 min. A.I.	4	14.6
			8 min. A.I.	5	13.9
			13 min. A.I.	6	12.8
			23 min. A.I.	7	12.7
			33 min. A.I.	8	13.8
			48 min. A.I.	9	13.0
2	Veeman Burke Belle	C-B-G	B.I.	1	9.8*
			150 cc. I.C.	2	11.1
			300 cc. I.C.	3	13.3
			3 min. A.I.	4	12.0
			8 min. A.I.	5	11.3
			17 min. A.I.	6	10.6
			23 min. A.I.	7	10.5
			28 min. A.I.	8	10.6
			33 min. A.I.	9	10.8
			38 min. A.I.	10	10.4
			43 min. A.I.	11	10.2
			60 min. A.I.	12	9.7
3	Veeman Belle	C-B-G	B.I.	1	10.2*
			150 cc. I.C.	2	13.2
			300 cc. I.C.	3	14.8
			3 min. A.I.	4	12.4
			8 min. A.I.	5	12.1
			13 min. A.I.	6	11.7
			18 min. A.I.	7	11.6
			23 min. A.I.	8	11.5
			28 min. A.I.	9	11.3
			33 min. A.I.	10	11.5
			43 min. A.I.	11	11.4
			58 min. A.I.	12	11.5
4	Sensation Veeman	C-B-S-G	B.I.	1	10.1*
			150 cc. I.C.	2	11.7
			300 cc. I.C.	3	15.5
			5 min. A.I.	4	13.5
			10 min. A.I.	5	13.7
			15 min. A.I.	6	12.6
			21 min. A.I.	7	12.3
			26 min. A.I.	8	13.0
			32 min. A.I.	9	12.3
			41 min. A.I.	10	11.1
			51 min. A.I.	11	11.0
			62 min. A.I.	12	10.9
5	Sensation Veeman	C-B-S-G	B.I.	1	9.9*
			150 cc. I.C.	2	12.3
			300 cc. I.C.	3	15.2
			7 min. A.I.	4	14.8
			12 min. A.I.	5	15.0
			18 min. A.I.	6	15.4
			25 min. A.I.	7	16.0
			29 min. A.I.	8	13.8
			39 min. A.I.	9	13.6
			50 min. A.I.	10	13.4
			59 min. A.I.	11	13.0
			71 min. A.I.	12	13.0

B.I. = Before Injection; this determines the normal level.

I.C. = Injection Complete; blood drawn after this volume of solution had been injected.

A.I. = After Injection; this is the elapsed time between the completion of injections and the drawing of samples.

* = The normal, or pre-injection, reading taken as zero in figure 3.

During this investigation, cows suffering from hypocalcemia were not available, so the determinations were all made on normal lactating cows. There is little reason to doubt, however, that C-B-S-G solution would be more efficient than C-B-G solution in elevating and maintaining the serum calcium level in hypocalcemic cows as it is in normal cows. This is another problem for investigation.

CONCLUSIONS

1) Boric acid increases the solubility of calcium d-saccharate in water.

2) Boric acid, in solution, reacts with calcium d-saccharate to form what is probably a new compound to which the name calcium boro-saccharate has been given.

3) A solution containing 10 parts boric acid, 37.5 parts calcium d-saccharate and 100 parts calcium d-gluconate in 500 parts distilled water is physically stable and is less toxic for rabbits and no more toxic for cows, when injected intravenously, than a commercial solution of calcium boro-gluconate.

4) The intravenous injection of either of these solutions into cows causes an immediate marked rise in the serum calcium level. This rise is followed by a rapid drop, and, in the case of the C-B-S-G solution, there is a secondary rise about one-half hour after injection.

References

[1]Little, W. L., and Wright, N. C.: Etiology of Milk Fever in Cattle. Brit. J. Expti. Path., *6*, (1925): 129.

[2]Newton, O. M.: The Calcium Content of the Blood of Cattle. Rev. Med. Vet., *16*, (1934): 12.

[3]Fraix, E.: Intravenous Calcium Therapy. Semana méd. (Buenos Aires), *1*, (1937): 835.

[4]Rogers, J. A.: Field Experiences in the Use of Calcium Gluconate. J.A.V.M.A., *31*, (1931): 555.

[5]Carratala, R. E.: Calcium in Toxicology. O Hosp., (Rio de Janeiro, Brazil), *19*, (1941): 191.

[6]DeCarli, F.: The Solubility of Calcium Gluconate. Ann. Chim. applic., (Rome, Italy), *21*, (1931): 447.

[7]Dryerre, H., and Greig, J. R.: The Specific Chemo-Therapy of Milk Fever by the Parenteral Administration of Calcium Boro-Gluconate. Vet. Med., *30*, (1935): 234.

Studies on Bovine Mastitis
I. A Note on Clavacin Therapy in Bovine Mastitis

E. H. PETERSON, Ph.D., D.V.M., and ROBERT GRAHAM, B.S., D.V.M.
Urbana, Illinois

DURING the past few years, much interest has been devoted to studies of antibacterial metabolic products of various fungi. Among those studies has been a derivative of the mold *Aspergillus clavatus*.[1,2,3] The material has been prepared in crystalline form and is known as clavacin.* Recently,[4] clavacin has been shown to be identical with patulin, an antibacterial substance produced by the mold *Penicillium patulum*. Patulin is a powerful bactericide possessing a considerable degree of toxicity to mice. In preliminary trials, patulin was shown to have promise in the treatment of the common cold.[5].

An opportunity has been afforded to infuse refined and unrefined clavacin experimentally as a therapeutic agent in the treatment of bovine mastitis. The unrefined clavacin consisted of the supernatant fluid (pH 4.5) obtained from the mold cultures after Seitz filtration. Assay has shown crude clavacin to contain approximately 1 Gm. of active substance per liter. The initial observation to be reported consisted of injecting three quarters of 1 cow, respectively, with 5 cc., 10 cc., and 15 cc. of unrefined clavacin. After twenty-four hours, the teats of all three treated quarters were firm and swollen. There was evidence of glandular irritation only in the quarter receiving the 15-cc. injection. After forty-eight hours, however, a severe, diffuse and persisting glandular inflammation developed in all quarters, being most severe in the quarter receiving the 5 cc. infusion. Milk secretion ceased and a state of active inflammation persisted for about ten days. The process gradually became chronic but had resulted in a very marked proliferation of connective or fibrotic tissue in all three quarters. The function of these quarters had plainly been severely damaged if not completely destroyed.

The four quarters of a second experimental cow were each injected with 15 cc. of crude clavacin (pH 4.5), the portions being diluted, respectively, with 0 cc., 15 cc., 30 cc., and 45 cc. of distilled water. The results closely paralleled those of the first trial, three of the quarters being badly damaged.

In a third series of udder infusions, the previously described injections were repeated in 2 other cows but with the modification that the pH of the crude clavacin was adjusted to the end point of bromthymol blue (approximately 7). The irritating effects upon the mammary gland were much reduced by the alteration in the pH, but still the injections resulted in perceptible increases in fibrotic tissue in all infused quarters, as judged by palpation. In further trials, two quarters of 1 cow used previously were injected, respectively, with 500 cc. and 300 cc. of a 1:5 dilution of clavacin (pH 7) in distilled water. Both quarters were acutely in-

From the Department of Animal Pathology and Hygiene, University of Illinois, Urbana.

*Clavacin used in these studies was made available through the courtesy of Dr. H. W. Anderson of the Horticulture Department and Dr. H. E. Carter of the Department of Chemistry, University of Illinois.

(*Continued from page* 315)

[9]Pasternack, R., and Giles, W. R.: Stable Calcium Gluconate Solution and Process for Making Same. U. S. Patent 1,965,535, July, 1934.

[10]Godden, W., and Duckworth, J.: Variations in Serum Magnesium and in the Partition of Serum Calcium in Normal Parturition and in Milk Fever. Biochem. J., *29*, (1935): 445.

[10]Glaser, F.: Physiological Basis for Intravenous Calcium Therapy. Deutsche med. Wchnschr., *52*, (1926): 1336.

[11]Kramer, B., and Tisdall, F. F.: A Simple Technique for the Determination of Calcium and Magnesium in Small Amounts of Serum. J. Biol. Chem., *47*, (1921): 475.

flamed in twenty-four hours; the inflammation persisted for about two weeks and resulted in marked induration. This cow subsequently freshened in about two months. The lacterifus sinusi of all clavacin-treated quarters were partially, if not completely, closed, presumably due to fibrosis, for while the quarters were distended with milk, little or no fluid could be withdrawn from the teats.

The udders of 2 cows were injected with 1:10,000 dilutions of crystalline clavacin in distilled water and in physiologic saline. Two quarters of one cow were infused, respectively, with 250 cc. and 400 cc. of clavacin, using distilled water as the diluent. The quarters being inactive and small were filled to capacity. There resulted a semiacute inflammation of both treated glands accompanied by marked induration. One quarter of a second cow was infused with 500 cc. of a similar preparation. The udder in this case was much larger than in the previous cow. The reaction was never at any time acute, but did result in a pronounced proliferation of connective tissue. A second quarter of the same cow was infused with 500 cc. of a 1:10,000 dilution of clavacin in physiologic saline. The result closely paralled the reaction described.

DISCUSSION

The 5 cows used in these trials were old animals near the end of lactation or dry. Of the 19 quarters treated, 17 showed the presence of more or less marked fibrosis. Most of them were shedding streptococci.

Streptococci were not eliminated from any of the infected quarters by the clavacin infusions. These observations, however, were limited and the negative results would not in themselves eliminate clavacin as a possible therapeutic agent in the treatment of mastitis. Administrations of clavacin, however, which did not even temporarily reduce the streptococcal flora of infected glands proved highly irritating to the tissues and resulted in marked proliferation of connective tissue. It is felt, therefore, that the infusion of unrefined and refined crystalline clavacin has little possibility as a therapeutic agent in the treatment of bovine mastitis.

SUMMARY AND CONCLUSIONS

Nineteen of the quarters of 5 cows were infused with various concentrations of crude and purified clavacin. Most of the quarters were shedding streptococci. In every instance, unrefined and refined clavacin proved to be irritating as indicated by connective tissue proliferation with or without symptoms of acute inflammation. The induced fibrosis was so marked in some cases as to destroy the function of the gland. In one cow which freshened subsequent to clavacin administration, the milk sinuses were so blocked by fibrotic tissue that little or no milk could be withdrawn. Infusions of clavacin did not, at the same time, materially affect the numbers of streptococci being eliminated in the secretion from the respective quarters. On the basis of limited observations, clavacin proved unsatisfactory as a therapeutic agent in the treatment of bovine mastitis by udder infusion.

References

[1]Wiesner, B. P.: Bactericidal Effects of *Aspergillus clavatus*. Nature, *149*, (1942): 356.

[2]Waksman, Selman A., Horning, Elizabeth S., and Spencer, Ernest L.: The Production of Two Antibacterial Substances, Fumigacin and Clavacin. Science, *96*, (1942): 202.

[3]Waksman, Selman A., Horning, E. S., and Spencer, E. L.: Two Antagonistic Fungi, *Aspergillus fumigatus* and *Aspergillus clavatus*, and Their Antibiotic Substances. J. Bact., *45*, (1943): 233.

[4]Hooper, I. R., Anderson, H. W., Skell, P., and Carter, H. E.: The Identity of Clavacin and Patulin. Science, *99*, (1944): 16.

[5]Raistrick, Harold, S. C. D. Camb., F. R. S., and Collaborators: Patulin in the Common Cold. Collaborative Research on a Derivative of *Penicillium patulum* Bainier. The Lancet, *245*, (Nov. 20. 1943): 625-635.

Isolation of the Bacillus Anthracis from Spinose Ear Ticks Ornithodorus Megnini

GEORGE W. STILES, M.D., Ph.D.
Denver, Colorado

ACCORDING to our laboratory records, sporadic outbreaks of anthrax in cattle have occurred for many years in restricted areas of Colorado. Less frequently the disease has appeared in swine and rarely in sheep.

HISTORY

On September 22, 1943, Dr. J. Gaylord Varner, veterinarian of Trinidad, Colorado, submitted a cow's ear for confirmatory diagnosis. Clinically, the sudden death of the cow in a herd where anthrax had recently been established strongly suggested that the animal had died of anthrax. When received, the ear specimen was in excellent physical condition.

Stained blood smears from the ear veins showed myriads of rods resembling anthrax bacilli. After seeding cultures and securing a few loops of blood for guinea pig inoculation, further examination of the inner portion of the ear revealed a colony of spinose ear ticks (*Ornithodorus megnini*).

LABORATORY FINDINGS

The blood from the cow's ear proved to be positive for anthrax, both by cultural methods and guinea pig inoculation. The test guinea pig died within forty-eight hours. Autopsy showed the usual splenic enlargement, edematous swelling at the site of injection, and *Bacillus anthracis* was recovered on culture. This confirmed the clinical diagnosis of anthrax in the cow.

Perhaps the laboratory's routine duty was fulfilled when a diagnosis of anthrax was established in the dead cow. However, the discovery of spinose ear ticks in the ear of this cow dead of anthrax raised the question as to the possibility that these ticks might also carry anthrax bacilli, either on the outside of or within their bodies. About one dozen larvae and nymphs were removed from the deep recesses of the anthrax infected cow's ear. They were washed one hour in tap water, briefly immersed in 70 per cent alcohol, and then placed into boiling water for a few seconds. The cleansed ticks were finally rinsed in sterile broth, which on culture proved negative for anthrax bacilli, thus suggesting the probable absence of anthrax organisms on the exterior of the ticks.

Subsequently, the treated ticks were macerated with sterile saline solution in a sterile mortar. A single anthrax colony developed on each of two agar tubes seeded from the tick suspension (fig. 1).

A medium sized guinea pig injected subcutaneously with 1 cc. of the tick suspension died in three days. *B. anthracis* was recovered from the heart blood and the enlarged spleen.

Cultures from the spinose ear ticks were further identified by our Washington laboratory, reported by Giltner,[1] who stated that the cultural characteristics on diagnostic mediums and guinea pig inoculation resulted in a positive diagnosis of anthrax.

Engorged nymphal ticks from two additional anthrax infected cow's ears failed to show evidence of anthrax bacilli.

In the first instance, 2 ticks only were used, while in the second, 4 specimens were recovered from the ears of cows dying from anthrax. These ticks were washed, treated in 70 per cent alcohol, and immersed briefly in boiling water, as were the dozen larvae and nymphs in the first experiment. Cultures from the macerated ticks and guinea pig injections resulted negatively for anthrax.

These negative findings are explained by Bishopp's[2] description of the life history of the spinose ear ticks. Apparently spinose ear ticks feed and engorge on the host only during their larval and nymphal stages.

From the Branch Pathological Laboratory, Bureau of Animal Industry, United States Department of Agriculture, Denver.

Bishopp states, "This is the only known species of tick which does not feed in the adult stage, the adults dying after oviposition is complete."

Since the life history of the spinose ear tick is well known to entomologists, the reader is referred to published articles on this phase of the subject. Spinose ear ticks are widely distributed throughout the arid and semiarid parts of our country.

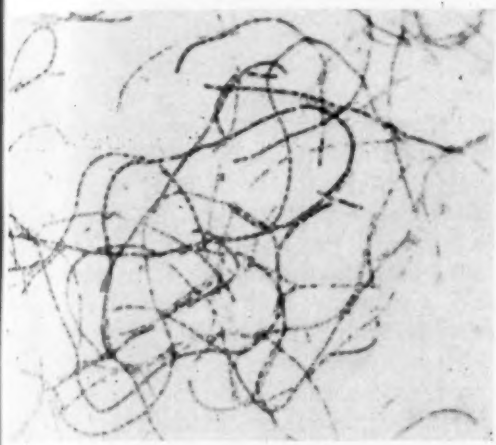


Fig. 1—Bacillus anthracis-stained organisms showing chain and spore formation. Two day agar culture recovered from spinose ear ticks. x1000.

Possible Injury Caused by Spinose Ear Ticks

While spinose ear ticks are most frequently found in the ears of cattle, other animals may also be infested. Imes[3] states that this parasite may be found in horses, dogs, jackrabbits, and sheep. Under favorable conditions, spinose ear ticks may attack other mammals, including man. My own observations include finding these ticks in cattle, dogs, cats, and coyotes; in one instance, on the same premises where cats and dogs were infested, a man was found harboring these ticks.

Serious damage to livestock is reported by these pests in areas where they are abundant. The parasites injure the tender inside skin of the ears, resulting in ulceration, and a condition commonly known as "ear canker." Heavy infestation may cause deafness, lowered vitality, lessened milk production, nervousness, unthriftiness, and sometimes death to aged animals during severe weather.

Bishopp[2] says he knows of no instance where anthrax organisms have been isolated from spinose ear ticks, and he raises the question whether they could be successfully passed from the nymphal stage through the adult and the egg to the next generation. The answer to this inquiry would have an important bearing on the spread, longevity, and ultimate destruction of anthrax organisms from animals dead of this disease. Herms[4] advises that he has searched his reprints on ticks but failed to find any literature pertaining to the transmission of anthrax by these parasites.

Since this species of tick can survive many days in nature, under favorable climatic conditions, spinose ear ticks if not immediately destroyed by burning, deep burying, or other adequate means, could become one means of disseminating anthrax bacilli.

The systematic treatment of animals infested with spinose ear ticks is urgently recommended to livestock owners.

Summary

1) *Bacillus anthracis* was isolated from a colony of spinose ear ticks, in various stages of development, attached to the ear of a cow dead of anthrax.

2) Two negative findings are reported in engorged nymphs from cows dead of anthrax. Entomologists report that this species of tick feeds only during the larval and nymphal stages.

3) The positive finding indicates that spinose ear ticks from anthrax carcasses may possibly spread the disease to healthy animals. Tick infested carcasses of animals dead of anthrax should be promptly disposed of.

4) Greater effort should be made by livestock owners to keep their herds free from spinose ear ticks because of the possibility of spreading disease.

References

[1]Giltner, L. T.: Personal Communication. Nov. 6, 1943.

[2]Bishopp, F. C.: Personal Communication. Dec. 10, 1943.

[3]Imes, Marion: The Spinose Ear Tick. Farmers' Bul. 980, BAI, USDA, 1918.

[4]Herms, W. B.: Personal Communication. Oct. 19, 1943.

Studies on Johnin
V. Producing and Standardizing a Potent Product

HOWARD W. JOHNSON, B.S., M.S., D.V.M.
Auburn, Alabama

THE TECHNIQUE usually followed in the production of tuberculin and johnin consists in (1) maintaining the strain or strains of organisms used in such production on a solid culture medium, (2) transferring cultures from this solid medium to a satisfactory liquid medium for the production of "seed" cultures, (3) transferring these seed cultures to flasks or bottles of a satisfactory liquid medium and incubating these until approximately maximum growth has occurred, and (4) preparing tuberculin or johnin from the filtrates of these cultures. It is generally assumed that growth in these inoculated flasks or bottles will follow a definite course as described by Lane-Claypon,[1] Buchanan,[2] and others as a succession of growth phases. These, five in number, are called the initial stationary phase, the lag phase, the logarithmic phase, the negative growth acceleration phase, and the maximum stationary phase.

The chief limiting factors found by the author in his early attempts to produce johnin were (1) the inability of strains of *Mycobacterium paratuberculosis* used to establish surface growth on most synthetic mediums and (2) the marked tendency for the amount of growth to diminish as series transfers of a culture were made on a single medium.

Studies and observations reported in this paper were made in attempts to find a satisfactory method of producing and standardizing a potent johnin. They include (1) efforts to devise a satisfactory solid medium for use in the cultivation and maintenance of laboratory strains of *M. paratuberculosis*, (2) attempts to devise a satisfactory liquid medium for growing seed cultures of this organism, (3) measurement of the rate and amount of growth of different strains of *M. paratuberculosis* on Dorset and Henley's synthetic medium, and (4) efforts to determine if any relation exists between the rate and quantity of such growth, the accompanying pH changes in the medium, the amount of trichloracetic acid precipitate obtained from johnins prepared weekly from such mediums, the chicken potency tests of such johnins, and the response elicited by the same johnins injected simultaneously into the skin of sensitized cattle.

From Regional Animal Disease Research Laboratory, Bureau of Animal Industry, Agricultural Research Administration, United States Department of Agriculture, Auburn, Ala.

SOLID MEDIUM STUDIES

All strains of *M. paratuberculosis* used in these experiments were grown on solid mediums for at least two years prior to the time of these studies. All cultures were incubated at 38 C.

Some of the egg, serum and agar, and agar-base synthetic mediums which have been suggested for the cultivation of *M. paratuberculosis* or *M. tuberculosis* were compared with modifications of Dorset and Henley's[3] synthetic medium for use in growing stock cultures of *M. paratuberculosis*. (*see* table 1.)

All synthetic agar mediums were made with 4, 7, and 10 per cent glycerine. The 4 per cent glycerine types always produced somewhat better growth, although both the 7 and 10 per cent glycerine types were nearly as satisfactory.

All extracts of acidfast organisms used were prepared after the method employed by Minett.[8] Ten Gm. of dried culture were mixed with 60 cc. of distilled water and 40 cc. of glycerol, then steamed for one hour. When this extract was employed, it comprised 10 per cent of the volume. All egg

mediums contained approximately two-thirds egg.

A satisfactory bovine lymph gland medium, which is a modification of Dorset and Henley's[3] synthetic medium, was prepared as follows:

l-asparagine	14.0 Gm.
Dipotassium phosphate c.p. ($K_2HPO_4+3H_2O$)	1.8 Gm.
Sodium citrate c.p. ($Na_3C_6H_5O_7+2H_2O$)	0.9 Gm.
Magnesium sulphate, U.S.P. ($MgSO_4+7H_2O$)	1.5 Gm.
Ferric citrate, U.S.P., VIII (Scales)	0.3 Gm.
Dextrose, U.S.P.	10.0 Gm.
Glycerine c.p. (U.S.P.)	40.0 Gm.
Mesenteric lymph glands (calf) fat-free and ground	100.0 Gm.
Agar Agar	20.0 Gm.
Water (tap) to make	1000.0 cc.

The inorganic salts, dextrose, and agar were dissolved separately in cold tap water. The asparagine was dissolved in warm tap water and the ferric citrate in hot tap water. All materials were added to the asparagine solution in the order listed, with constant stirring. The entire mixture was then brought to a boil, allowed to cool slightly, and tubed in approximately 10 cc. amounts in screw-top vials. These tubes were then sterilized at 12 pounds pressure for twenty-five minutes and slanted. Only medium not more than ten days old was used.

The lymph glands from young cattle (about 1 year) in good condition were found to be the more satisfactory for use in the lymph-gland medium. All the connective tissue and fat possible were first removed from around the glands and then they were blended in a high speed blender before being added.

Lymph-gland medium (No. 7, table 1) was found to support a rapid and marked growth, was simple in preparation, and held moisture well, even when kept in the incubator (38 C.) for six to ten months. Studies are now in progress to compare the value of this medium with that of others for the primary isolation of *M. paratuberculosis*.

Laboratory strains of the organism usually grew well on this lymph-gland agar. (*see* fig. 1.) Transfers to liquid mediums

TABLE 1—Solid Culture Mediums Tested

No.	Chief Ingredients	Authors	Growth
1	Egg liver extract	[6]Dunkin (1928) [7]Lange and Pescatore (1937) [8]Minett (1942)	+ + +
2	Egg potato broth	[8]Minett (1942)	+ +
3	Egg veal broth	[8]Minett (1942)	+
4	Agar, serum, extract of *M. tuberculosis*	[9]M'Fadyean, *et al.* (1912)	+ +
5	Agar, serum, tuberculin	[9]M'Fadyean, *et al.* (1912)	+ +
6	Egg tuberculin	[9]M'Fadyean, *et al.* (1912)	+
7	Synthetic medium, calf lymph glands	Modified Dorset and Henley[3]	+ + + +
8	Synthetic medium, killed phlei	Modified Dorset and Henley[3]	+ +
9	Synthetic medium, killed phlei, calf lymph glands	Modified Dorset and Henley[3]	+ + + +
10	Egg phlei	[11]Twort (1910) [10]Sheather (1914)	+
11	Egg *M. paratuberculosis*	[11]Twort (1910) [10]Sheather (1914)	+
12	Synthetic medium, blood charcoal calf lymph gland	Modified Dorset and Henley[3]	+ + + +
13	Synthetic medium, agar	Modified Dorset and Henley[3]	+ +
14	Synthetic medium, (Long) phlei extract	[12]Wright (1928)	+ +
15	Synthetic medium, Vitamin K	[13]Woolley and McCarter (1940)	+ +
16	Synthetic medium, Bovine intestinal mucosa	Modified Dorset and Henley[3]	+ + +

+ = Very slight growth; + + = Slight growth; + + + = Moderate growth; + + + + = Marked growth.

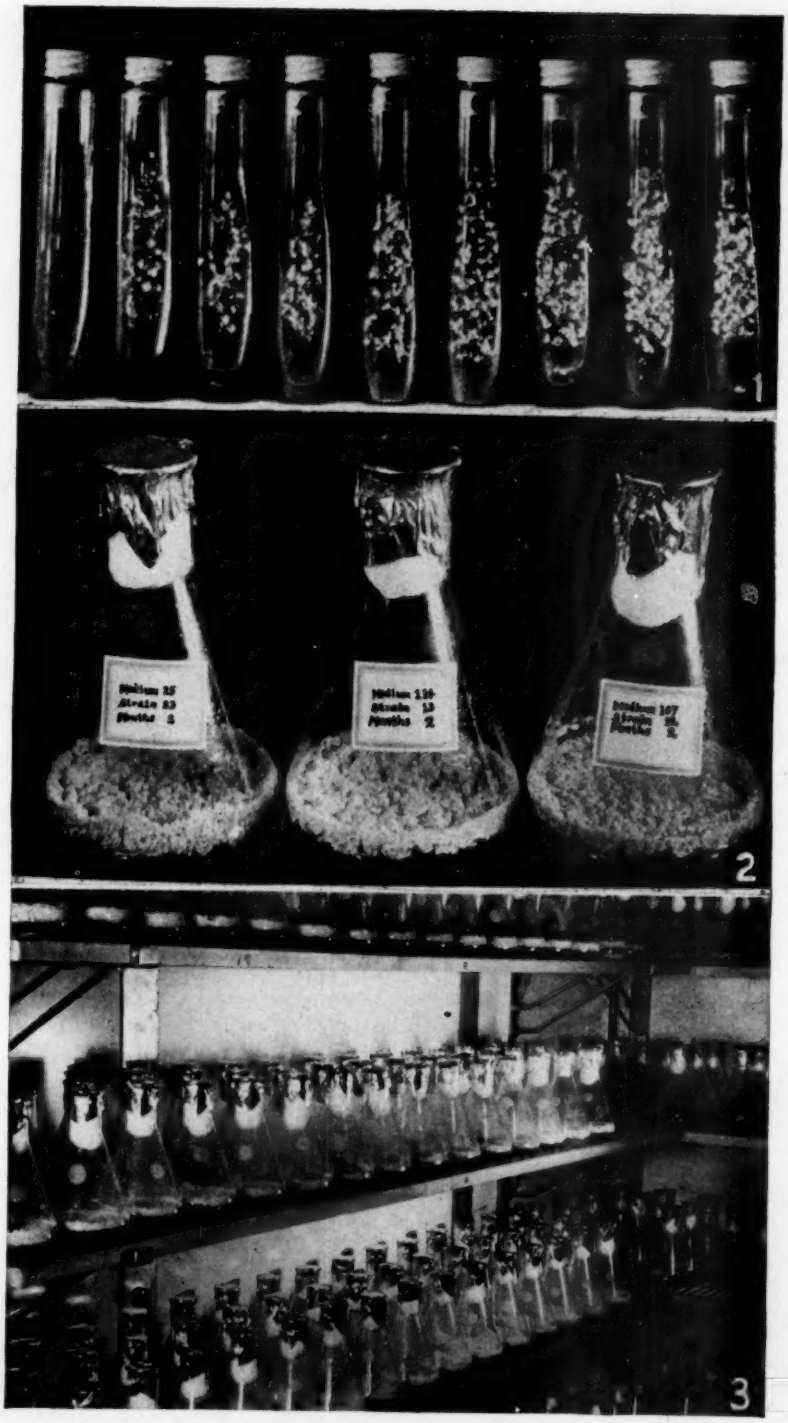


Fig. 1—Mycobacterium paratuberculosis (0, 1, 2, 3, 4, 5, 6, 7, and 8 weeks) growth on lymph gland medium.
Fig. 2—Three strains of Mycobacterium paratuberculosis growing on 3 synthetic mediums.
Fig. 3—Mass cultivation of Mycobacterium paratuberculosis on Dorset and Henley's[3] synthetic medium.

were easily made when vigorous growth had taken place, four to six weeks after inoculation, before the cultures were too old and hard.

LIQUID MEDIUM STUDIES

One hundred eighty-seven mediums were prepared, inoculated, and observed in an effort to devise a liquid medium satisfactory for growing "seed culture" for inoculation of Dorset and Henley's[3] synthetic medium from which a satisfactory intradermic johnin could be produced. Six mediums, which are modifications of Dorset and Henley's[3] synthetic medium, were found to initiate growth more rapidly when cultures were transplanted from solid mediums and also to produce a greater volume and uniformity of growth.

The formula for these six mediums, which are now used in this laboratory for the preparation of seed cultures, is as follows:

No. 5 =

l-asparagine	14.0 Gm.
Dipotassium phosphate c.p. ($K_2HPO.3H_2O$)	1.8 Gm.
Sodium citrate c.p. ($Na_3C_6H_5O_7.2H_2O$)	0.9 Gm.
Magnesium sulphate, U.S.P. ($MgSO.7H_2O$)	1.5 Gm.
Ferric citrate, U.S.P., VIII (Scales)	0.3 Gm.
Dextrose, U.S.P.	10.0 Gm.
Glycerine, c.p. (U.S.P.)..	70.0 Gm.
Phlei extract (Minett[8])..	100.0 Gm.
Water (tap) to make.....	1000.0 cc.

No. 25 = No. 5 medium in which 10 Gm. of yeast extract was substituted for the Phlei extract.

No. 111 = No. 5 medium except that 0.9 Gm. of ammonium citrate c.p. crystals was substituted for 0.9 Gm. of sodium citrate and the Phlei extract omitted.

No. 114 = No. 5 medium except that 0.9 Gm. of sodium succinate (pure) was substituted for 0.9 Gm. of sodium citrate and the Phlei extract omitted.

No. 167 = No. 5 medium except that 2.0 Gm. of ammonium urate c.p. (acid) was substituted for 0.9 Gm. of sodium citrate and the Phlei extract omitted.

No. 186 = No. 5 medium except 0.2 per cent of vitamin K was added.

All mediums were adjusted to a pH of 6.5 to 6.6. Then 100 cc. portions were placed in 300 cc. Erlenmeyer flasks and sterilized. If they were heated too long or at too high a temperature, they became dark and unfavorable for growth. Autoclaving at 12 pounds pressure for twenty-five minutes has been found to be sufficient for sterilization. The mediums were, however, removed from the autoclave as soon after the lapse of 25 minutes as the lowering of the pressure allowed.

It was observed that different strains have a preference for one or sometimes two of the above mediums and that there was less tendency for strains to gradually produce less and less growth on liquid mediums if they were subcultured upon a medium of their liking. If such strain tendencies were taken into consideration in selecting seed culture mediums, the cultures usually made profuse uniform growth (2 or more Gm. dry weight) in one to two months. (*see* fig. 2.)

GROWTH AND JOHNIN POTENCY STUDIES

There was no one of the six mediums described in this paper which served equally well for initiating and perpetuating growth of all cultures tried. Three of these (No. 114, 167, 25) were chosen for the propagation of 8 seed cultures used in the growth studies reported in this paper. When these seed cultures were in "full sweat" (3 to 8 weeks old), transfers were made from them to Dorset and Henley's[3] synthetic medium for *M. tuberculosis*. A group of 200 or more 300 cc. Erlenmeyer flasks was inoculated simultaneously for each lot of johnin produced with each of the 8 strains under study. Growth was usually uniform and satisfactory. (*see* fig. 3.)

Two weeks after inoculation and for four to thirteen successive weeks thereafter, 3 flasks (a sublot) from each group, which had been selected at random at time of inoculation, were removed from the incubator, and used in the following studies.

The pH of the medium from each of 3 flasks harvested each week was measured with a Hellige pH meter employing a quinhydrone and saturated KI bulb type glass electrode.

The flasks were then steamed in an Arnold sterilizer for three hours. The organisms which had grown on the medium were collected on tared, filter paper by filtration and dried in vacuum over sulphuric acid (c.p.) for seventy-two hours. The average weight of organisms per flask was determined and recorded.

Intradermic johnin was made from this filtrate, using the Dorset and Henley[3] technique for preparing tuberculin. Each weekly sublot was evaporated by heat to 20 per cent of the original volume of the medium and an equal volume of 1 per cent phenolated saline added (25% O.J.[5]) regardless of the weight of organisms removed from the 3 flasks. The 170 or more remaining flasks were harvested after a maximum of ten weeks and 25 per cent O. J. johnin prepared for field and chemical fractionation studies.

The amount of trichloracetic acid precipitate in each sublot of johnin was determined by mixing 10 cc. of the johnin with 2.5 cc. of a 40 per cent solution of trichloracetic acid in a 15 cc. Pyrex centrifuge tube graduated in 0.1 cc. divisions and centrifuging for five minutes at 1,700 r.p.m. Direct readings of the amount of precipitate were made and recorded.

The chicken potency titer of each sublot was determined by testing 4 or more dilutions of each sublot, each on 5 different and previously sensitized chickens. The technique employed was described in the second paper of this series.[5]

After the four to thirteen weekly sublots of johnin had been made from each culture being studied, their bovine potency titers were determined by injecting, simultaneously, dilutions of each of the sublots into the skin of cattle previously sensitized with the strain of *M. paratuberculosis* used in making these sublots of johnin. The

FIGURE 4

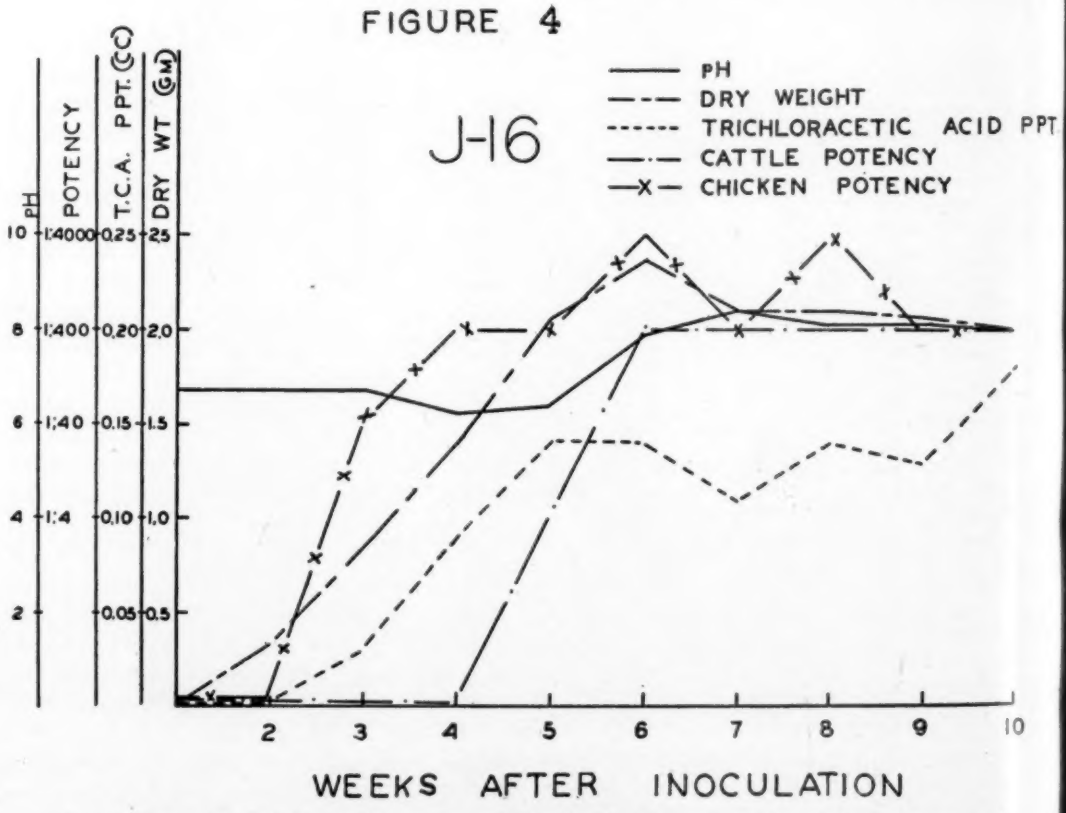


Fig. 4—Curves formed by satisfactory growth of Mycobacterium paratuberculosis on Dorset and Henley's[3] synthetic medium.

procedure for sensitizing and multiple testing cattle was described in an earlier paper of this series.[4] The highest dilution which produced a satisfactory reaction was recorded as the potency titer.

Curves were plotted from the data obtained from each series of sublots harvested from flasks which had been inoculated simultaneously with material from the seed cultures. These showed the weekly deviations of (1) the dry weight of organisms, (2) the pH of the medium, (3) the trichloracetic acid precipitate in 10 cc. of johnin prepared from this medium, (4) the chicken potency titers of such johnins and (5) the cattle potency titers of such johnins.

The maximum of pH, trichloracetic acid precipitate in 10 cc. of 0.25 per cent O.J., dry weight per flask, chicken potency, and cattle potency from the data resulting from 212 weekly sublots of 24 growth studies of 8 strains of *M. paratuberculosis*, are shown in table 2. Figures 4 and 5 were chosen as typical examples of the two types of growth curves observed in studying the characteristics of *M. paratuberculosis* growth on Dorset and Henley's[3] liquid medium. These show clearly the five growth phases of Lane-Claypon.[1] Figure 4 represents the more desirable and figure 5 the less desirable type of growth.

A sharp difference between the dry weight of resulting organisms in the unsatisfactory and satisfactory types of growths of *M. paratuberculosis* was not observed. The maximum dry weights of organisms from resulting medium in the 13 unsatisfactory lots of johnin varied from 0.33 Gm. to 2.72 Gm. per flask with an average of 1.83 Gm. per flask. The maximum dry weights of organisms from resulting medium in the 11 satisfactory lots of johnin varied from 2.00 to 2.90 Gm.

FIGURE 5

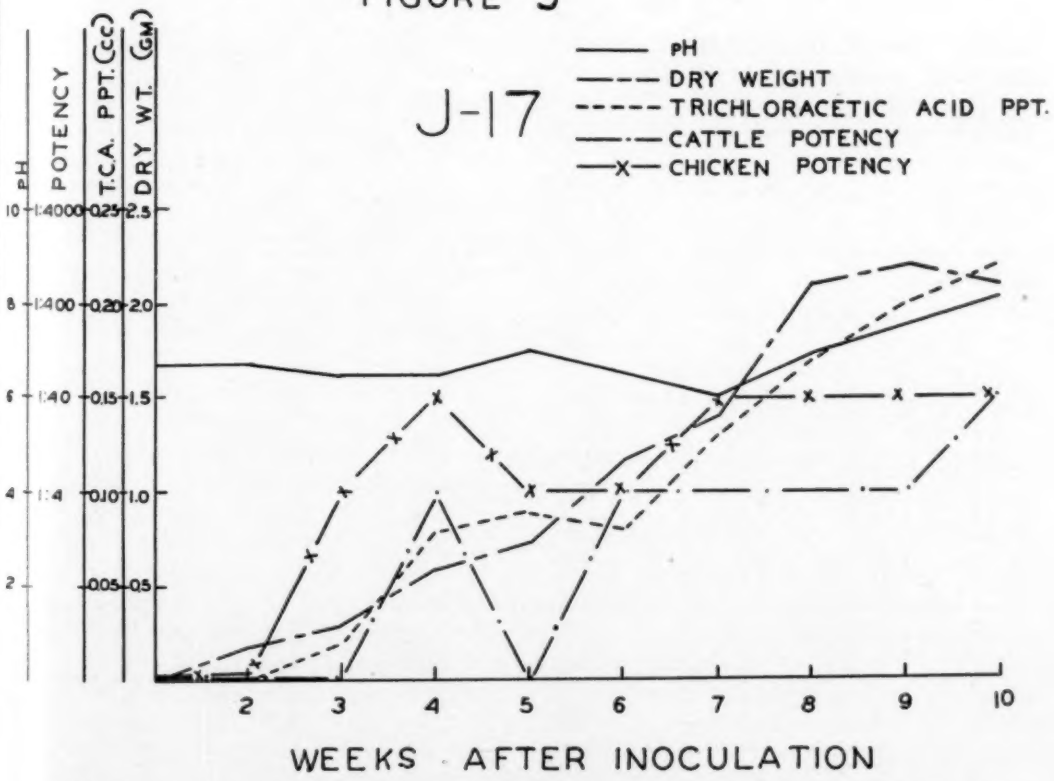


Fig. 5—Curves formed by unsatisfactory growth of Mycobacterium paratuberculosis on Dorset and Henley's[3] synthetic medium.

per flask with an average of 2.35 Gm. per flask.

The reaction of the synthetic medium used usually became more acid during the early logarithmic phase of the growth of *M. paratuberculosis*. Then, as acceleration of the negative growth and the maximum stationary phases were reached, the reaction of the medium became alkaline rapidly if the potency of the prospective johnin was to prove satisfactory. The maximum potency of the 11 johnins produced from the meduim on which a satisfactory growth curve had resulted was reached when the pH of the medium was 7.5 or above. The maximum pH was not above 7.5 in 10 out of 13 johnins in whose production an undesirable type of growth curve resulted. The one lot of "J" strain johnin which was not of satisfactory potency, had a very long lag phase but ultimately reached a pH of 7.5. The growth of "O" strain organisms on the medium from which the two lots of "O" strain johnin that reached high pH values (7.8 and 8.5) were produced, was smooth and hard rather than rough and soft as observed when strains produced satisfactory johnins.

When the pH went above 7.5 there was a tendency for the potency of the johnins to decrease slightly if incubation was continued.

The maximum amounts of trichloracetic acid precipitate in the johnins from the various lots showed an overlapping between the johnins of desirable and undesirable potencies. The range in the undesirable johnins was from 0.04 to 0.23 cc. for each 10 cc. of 25 per cent O.J. with an average of 0.1492. The range in the desirable johnins was 0.08 to 0.29 with an average of 0.1763.

It was noted also that with one exception, the third "N" johnin (table 2) which gave a suspicious reaction at 1 to 40 when tested on sensitized cattle, the undesirable johnins

TABLE 2—Growth Studies on Eight Strains of Mycobacterium Paratuberculosis on Dorset and Henley's Synthetic Medium, the Maximums of the Different Growth Factors That Were Being Studied and the Time at Which These Were Reached

		Maximum Reading and Week Obtained									
Johnin Lots by Strain Letter	Date Inoculated	pH.		T. C. A. ppt.		Dry weight per flask		Maximum Chicken Potency		Maximum Cattle Potency	
		pH.	Wk.	Cc.	Wk.	Gms.	Wk.	Dilution	Wk.	Dilution	Wk.
*A	5-41	6.6	0	Trace	10	0.36	10	§1-4	10	§1-4	10
G	10-41	7.5	7	0.10	8	2.36	8	†1-400	6	†1-400	9
*G	1-42	5.9	6	0.21	10	2.50	10	†1-4	10	†1-4	10
G	6-42	8.3	10	0.25	10	2.43	10	†1-400	9	†1-40	8
I	12-43	7.6	4	0.10	4	2.90	5	‡1-4000	4	†1-400	4
I	1-44	7.5	4	0.08	4	2.90	5	‡1-4000	4	†1-400	4
*I	5-41	7.8	9	0.19	8	2.72	8	†1-4	8	§1-4	10
J	10-41	8.1	9	0.29	8	2.46	6	†1-4000	7	‡1-4000	8
*J	4-42	6.8	3	0.04	4	0.33	7	‡1-4	7	§1-4	10
*J	12-41	7.5	10	0.16	10	2.60	10	†1-40	10	†1-4	10
J	9-43	8.4	10	0.22	10	2.20	9	†1-4000	9	‡1-4000	10
J	9-41	8.4	7	0.18	10	2.10	7	†1-4000	8	†1-400	6
J	2-42	7.9	9	0.20	10	2.10	7	‡1-4000	6	†1-400	7
*M	9-41	6.8	7	0.23	10	1.56	10	†1-4	10	§1-4	10
*M	11-41	7.3	9	0.20	10	2.00	10	†1-4	6	‡1-4	7
*M	12-41	7.2	5	0.12	8	2.20	9	†1-40	5	†1-4	8
*N	9-42	7.3	5	0.11	10	2.20	10	†1-4	5	‡1-4	8
N	11-42	7.7	5	0.17	7	2.00	7	†1-400	3	†1-400	6
*N	2-43	6.6	8	0.17	10	1.56	10	†1-40	7	‡1-40	9
*O	1-43	7.8	7	0.08	6	2.10	10	‡1-40	8	†1-4	9
*O	5-41	6.5	8	0.20	8	2.43	8	‡1-40	8	‡1-4	10
*O	6-42	8.5	10	0.23	9	1.26	10	†1-4	8	§1-4	10
R	8-42	8.2	8	0.25	10	2.20	8	†1-4000	5	†1-400	7
R	11-42	7.8	7	0.15	9	2.20	8	†1-400	3	†1-400	5

*Figure V type curves (unsatisfactory); †Positive; ‡Suspicious; §Less than.

failed to react in the 1 to 40 or greater dilutions, while the desirable johnins always reacted positively in the 1 to 40 dilution on sensitized cattle and with the exception of the third "G" johnin (table 2) in a much higher dilution.

It was further observed that the potency of the johnins during the early logarithmic phases of growth was greater when sensitized chickens were used for testing than when sensitized cattle were employed. The potencies indicated by both testing procedures were, however, in near agreement on johnins prepared after six or more weeks of incubation.

DISCUSSION

Dorset and Henley's[3] synthetic medium to which had been added 2 per cent agar and 10 per cent bovine lymph glands proved to be very satisfactory for transferring stock cultures of *M. paratuberculosis*. However, growth was always more rapid and abundant when fresh medium was used.

To obtain vigorous seed cultures of *M. paratuberculosis* on liquid mediums, it was found to be advantageous to transfer repeatedly (four to six weeks) each culture on one or more of the six modifications of Dorset and Henley's[3] medium which had been observed to manifest stimulation for that particular strain. When a culture was thus invigorated, transfers to the unmodified medium were made with more satisfactory results. Some strains, however, grew rapidly and retained a light, crumbly, rough growth, which was desirable, while others quickly became hard, waxy, and smooth, which proved to be unsatisfactory.

Although there was not always a close correlation between either the dry weight of organisms per culture flask from which johnin was prepared, the pH of the medium during the progress of the organisms' growth, or the amount of packed trichloracetic acid precipitate obtained from 10 cc. of intradermic johnin and the potency of the johnin, the dry weight of organisms per flask should reach 2 Gm., the pH 7.5 and the trichloracetic acid precipitate should yield from 0.15 to 0.25 cc. for each 10 cc. of 25 per cent O.J. intradermic johnin.

When growth was of the satisfactory type represented by figure 4, there was quite a marked similarity between the growth curves and the potency curves. The maximum of the cattle potency curve was reached at about the sixth week following inoculation and thereafter remained stationary or declined slightly. No such correlation between growth and potency was present when growth was of the unsatisfactory type (fig. 5). The representative curves (figures 4 and 5) were, therefore, not in agreement. The type of growth shown in figure 5 was relatively slower. The marked drop in potency at the fifth week in figure 5 was probably due to the small number of flasks (3) used in making the weekly lots of johnin. It is probable that the growth on the 3 flasks used that week had a somewhat longer lag phase and, therefore, had produced a less potent johnin.

The rapidity at which the growth reached the logarithmic phase seemed to affect markedly the final potency of the johnin. This was true even when the ultimate dry weight of organisms per flask and the trichloracetic acid precipitate in the johnin, prepared after ten weeks of incubation, were larger in the lots showing a marked lengthening of the lag phase. This was shown in figures 4 and 5. It was, therefore, desirable to obtain a growth that had a short lag phase and a logarithmic phase which produced a maximum growth at the end of five to six weeks of incubation to insure a johnin of high potency.

SUMMARY

1) A solid medium containing bovine lymph glands proved satisfactory for maintaining laboratory cultures of *Mycobacterium paratuberculosis*.

2) Cultures transferred from this solid medium to Dorset and Henley's synthetic liquid medium grew slowly and unsatisfactorily. Johnin made from such inoculated medium was of unsatisfactory potency.

3) Six different liquid mediums on which

cultures, transferred from the solid lymph-gland medium, grew vigorously were developed. Cultures were quite selective of the mediums on which their growth response was the greater. No one was satisfactory for all cultures.

4) Seed cultures which had been transferred several times on one or more of these "invigorating" liquid mediums would then grow for one, two, or three transfers to Dorset and Henley's liquid synthetic medium. Potent johnin was made from this inoculated synthetic medium.

5) The dry weight of organisms per culture flask and the trichloracetic acid precipitate per 10 cc. of intradermic johnin gave only relative estimates of the potency of the johnin, but were not in direct proportion to its potency.

6) The pH of the medium became more acid during the early logarithmic phase of the growth curve and then alkaline as the negative growth acceleration phase was reached.

7) A short lag phase and the production of maximum growth in five to six weeks after inoculation were found to yield more potent johnins.

ACKNOWLEDGMENT

The author wishes to acknowledge the many helpful suggestions of Mr. R. R. Henley of the Pathological Division, Bureau of Animal Industry, Agricultural Research Administration, U. S. Department of Agriculture, which have contributed greatly to these experiments.

References

[1]Lane-Claypon, Janet E.: Multiplication of Bacteria and the Influence of Temperature and Some Other Conditions Thereon. J. Hyg., *9*, (1909): 239.

[2]Buchanan, R. E.: Life Phases in a Bacterial Culture. J. Infect. Dis., *23*, (1918): 109.

[3]Dorset, M., and Henley, R. R.: A Synthetic Medium for *B. tuberculosis* with a Description of the Method of Producing Tuberculin. Biochemic Division, U. S. Bureau of Animal Industry, (Nov. 7, 1934).

[4]Johnson, Howard W.: Studies on Johnin, III. Multiple Testing on Sensitized Cattle as a Method of Studying Johnin Potency. Am. J. Vet. Res., *5*, (1944): 179-188.

[5]Johnson, Howard W., and Cox, B. F.: Studies on Johnin, II. A Proposed Method of Testing the Potencies of Johnin. Am. J. Vet. Res., *3*, (1942): 136-138.

[6]Dunkin, G. W.: A Diagnostic Agent for the Detection of Johne's Disease and Its Method of Preparation. J. Comp. Path. and Therap., *41*, (1928): 94-108.

[7]Lange, L., and Pescatore, H.: Beobachtungen über die Gewinnung und des Verhalten von Reinkulturen des Paratuberkulose-bazillus (Johneschen Bazillus). Z. Bakt., Abt. I, Orig., *140*, 1, 1937.

[8]Minett, F. C.: The Diagnosis of Johne's Disease of Cattle by Culture Methods. J. Path. and Bact., *54*, (1942): 209-219.

[9]M'Fadyean, J., Sheather, A. L., and Edwards, J. T.: Johne's Disease. J. Comp. Path. and Therap., *25*, (1912): 217-275.

[10]Sheather, A. L.: Johne's Disease. Vet. Rec., *27*, (1914): 276-281.

[11]Twort, F. W.: A Method for Isolating and Growing the Lepra Bacillus of Man (preliminary note). Pro. Royal Soc., Sec. B., *83*, (1910): 156-158.

[12]Wright, H. K.: Johnin as a Diagnostic Agent in Paratuberculosis. J.A.V.M.A., *72*, (1928): 1036-1060.

[13]Woolley, D. W., and McCarter, J. R.: Antihemorrhagic Compounds as Growth Factors for the Johne's bacillus. Proc. Soc. Exper. Biol., *45*, (1940): 357-360.

Brucellosis of Swine
II. Eradication by Blood Test and Segregation

H. S. CAMERON, D.V.M., M.S., Ph.D. and P. A. CARLSON, D.V.M.
Davis, California

BRUCELLOSIS of swine is increasingly important as an economic and public health entity. This station has been attempting experimentally, as an adjunct to controlled investigations, to eradicate the disease from field herds by raising potential breeding stock apart from the source of infection and by slaughtering the infected stock as replacements become available. This paper will describe the results.

The program adopted was, in general, as follows: The brood stock were blood tested by the tube technique; a reaction at 1:25 was considered positive. If infection existed, the entire group was handled as a positive unit, and no attempt was made to salvage nonreacting animals. (According to field observations,[1] when infection was present in a herd, the incidence was high.) The breeding program was continued, and only animals giving evidence of sterility were culled. Young pigs, potential brood stock, were segregated from the source of infection (the infected unit) at weaning. The stock for breeding were selected on the basis of the blood test as well as type. Only those negative to the test were bred; the others were consigned to the fattening lots.

In this report, only 2 herds are described in detail, although others are summarized. They have been selected because they represent two different types of management, and because in one the program came to a successful termination, whereas in the other it did not. Both herds, nevertheless, demonstrate the course of brucellosis in young pigs under natural exposure, and indicate that the infection can be eradicated in the manner described.

Herd 1.—This state herd had, for several years, produced pork for the institution's own use from garbage as well as grain. When it became necessary to expand considerably, a new hog unit was constructed about a mile from the old, and a new herd established. One hundred bred gilts were purchased in two groups of fifty each from a purebred breeder. The second group was delivered six months after the first. A blood test on the gilts at purchase revealed a high percentage of reactors. The records showed that group 2 had been vaccinated, but it was not determined what type of biologic was used. Table 1 gives the amount of infection and the farrowing data for each group.

TABLE 1—Agglutination and Farrowing Record of Purchased Gilts

GROUP	REACTORS* (%)	PIGS PER LITTER† (AVERAGE)
1	67.5	3.86
2	78.5	4.72

*Titers of 1 : 25 and over were considered positive.
†Based on pigs alive forty-eight hours after farrowing.

In view of the high incidence of infection, the value of the individuals, and the manager's willingness to coöperate in securing information on eradication methods, the program was adapted to the herd.

The quarters were ideally suited to segregation. A new farrowing barn with pens of metal pipe partitions and concrete floors was available. The infected unit was maintained in yards with concrete surface and adjoining pastures. At weaning, the young pigs were moved to comparable yards separated by a wide roadway. All quarters were well drained and easily disinfected. No garbage was fed to these young pigs. The personnel was ample in numbers but, with the exception of the paid supervisors, it was very unreliable.

This project is coöperative with the United States Bureau of Animal Industry. From the Division of Veterinary Science, University of California, Davis.

The first group segregated consisted of pigs from the 1942 fall crop. These were the progeny of the heavily infected foundation stock. The initial test was made on March 2, 1943, about two months after weaning, and before segregation was established. Table 2 shows the results of this test and subsequent ones.

TABLE 2—The Course of Infection in the 1942 Fall Pig Crop

Date	Number tested	Blood titer: Neg. at 1 : 25 No.	(%)	Pos. at 1 : 25 or over No.	(%)
Mar. 2, 1943	53 gilts	40	75.5	13	24.5
Apr. 5, 1943	67 gilts	53	79.2	14	20.8
	27 boars	23	85.2	4*	14.8
May 10, 1943	61 gilts	26	42.6	35	57.4
	23 boars	23	100.	0	0
May 28, 1943	62 gilts	17	27.4	45	72.6
Aug. 18, 1943	63 gilts	15	23.8	48	76.2
	11 boars	11	100.	0	0

*Removed from unit.

The break that occurred in May was undoubtedly from an individual pen exposure, since the boars were quartered in another yard and all remained negative, whereas the gilts showed evidence of heavy exposure. Conceivably, the personnel had established a contact between the single pen and the barn where infected stock were farrowing; fetal membranes or dead pigs may have been involved. Such carelessness had formerly been common in this herd.

Because of the high incidence of infection in the group, the gilts were placed with the infected unit. They were bred and farrowed with the foundation stock. The average litter size at weaning was 4.49. The boars, which were kept segregated, remained free from infection. Although brucellosis became widespread in the 1942 fall pig crop, data on the early testing indicated a low incidence of infection in weaned pigs despite exposure during the suckling period and that the break resulted from re-exposure and not from latent infection in the pigs.

An identical program was adopted in the 1943 spring pig crop. Table 3, showing the results on this group, clearly demonstrates the feasibility of the method.

TABLE 3—The Course of Infection in the 1943 Spring Pig Crop

Date	Number tested	Blood titer: Neg. at 1 : 25 No.	(%)	Pos. at 1 : 25 or over No.	(%)
Aug. 25, 1943	35 gilts	34	97.1	1*	2.9
	24 boars	24	100	0	0
Oct. 13, 1943	55 gilts	55	100	0	0
Oct. 27, 1943	9 boars	9	100	0	0
Nov. 18, 1943	82 gilts	81	98.8	1*	1.2
Jan. 14, 1944	66 gilts	59	89.4	7*	10.6
	13 boars	7	53.8	6	46.2
Jan. 31, 1944	45 gilts	25	55.5	20	44.5
	9 boars	5	55.5	4	44.5
Feb. 9, 1944	44 gilts	24	54.5	20	45.5

*Removed from unit.

Again the young pigs, weaned from infected sows and immediately segregated, were negative to the test, but acquired brucellosis from a subsequent exposure. The break occurred, as in the 1942 fall pigs, in one enclosure; and its magnitude indicated a heavy exposure, such as would result from an abortion within the group or from feeding on membranes or dead pigs.

TABLE 4—Summary of Agglutination Testing in Herd 2

Date	Stock*	Blood titers: Neg. at 1 : 25 No.	(%)	Pos. at 1 : 25 or over No.	(%)
Nov. 8, 1942					
Mature	(23)	6	26	17	74
Feb. 8, 1943					
Summer	'42 pigs(21)	9	42.8	12	57.2
Fall	'42 pigs(19)	19	100	0	0
June 19, 1943					
Fall	'42 pigs(25)	25	100	0	0
Aug. 17, 1943					
Fall	'42 pigs(16)	16	100	0	0
Spring	'42 pigs(11)	11	100	0	0
Oct. 4, 1943					
Fall	'42 pigs(11)	11	100	0	0
Spring	'43 pigs(14)	14	100	0	0
Jan. 7, 1944					
Fall	'42 pigs(13)	13	100	0	0
Spring	'43 pigs(14)	14	100	0	0
Mature	(11)	5	45.5	6†	54.5

*Figures in parenthesis refer to number tested.
†Four reacted at 1 : 25 only; the remaining two at 1 : 50.

Since most of the infected foundation stock had been retained as brood animals, an opportunity was available for further testing of this group. The results showed that titers were receding: when introduced, 67.5 and 78.5 per cent of the animals reacted at 1:25 in group 1 and 2, respectively, (*see* table 1), whereas eighteen months later in group 1 and twelve months later in group 2 the percentage of reactors at

1:25 or over was 12.5 and 41.3, respectively.

Herd 2.—Herd 2 was composed of purebred hogs having valuable blood lines. Normally the herd consisted of 25 to 30 brood sows. One objective of the owner was the sale of young pigs for breeding purposes. Our interest was attracted by an abortion storm following an abortion in a gilt that had been bred where purchased. A preliminary blood test of the brood stock showed 17 out of 23 reacting at 1:25 or over. Because this was a typical small farm herd, supervised by an owner who would follow the program implicitly, the herd was taken on for field observations. The tests are summarized in table 4.

That infection was actively spreading in the herd was clearly demonstrated by the positive reaction of the young pigs born in the summer of 1942 and allowed to remain in contact with the infected unit. The 1942 fall crop, on the other hand, was segregated at weaning; remained negative to the test, and are now in production.

Additional Herds.—Sufficient data were collected on other typical herds to show the course of infection in young pigs. Table 5 summarizes the information by showing: (1) the incidence of infection on the original test of brood stock, (2) the blood titers in pigs weaned at 1 to 2 months of age and therefore not exposed to brucellosis, and (3) the result of the latest test on the herd.

TABLE 5—A Summary of Reactors in Four Herds

Herd	Original Test Reactors		Weaned Pig Reactors		Last Herd Test Reactors	
	No.	(%)	No.	(%)	No.	(%)
3	52	74.3	3	2.9	18	26.9
4	22	55.0	3	1.3	14	38.9
5	12	91.0	0*	0		
6	10	37.7	5	5	21	29.1

*24 tested.

The only herd not improved was number 4. In this instance, a clean unit was established, but broke after abortion in a bred gilt purchased without benefit of test. In herds 3 and 6, all the reactors are in the mature stock and will be culled as soon as suitable replacements are available. Although the data on herd 5 are not complete, sporadic testing would indicate that the infection was not active.

Discussion

These two herds represent two types of management. In herd 1, the physical equipment with the exception of personnel was ideal for segregation. In herd 2, on the other hand, the equipment was makeshift, but the personnel thoroughly competent and reliable. In the latter herd, success was attained when the residual infection in the mature stock was culled. That a comparable termination was not reached in herd 1 reflects on the efficiency of the segregation and not on the principles of the eradication program. Both herds show, nevertheless, that the majority of weaned pigs are free from infection. Additional data on ten herds support this conclusion first observed by Howarth and Hayes.[2] Not all these herds were freed from the disease, but all furnished evidence in favor of the program. Failure to eradicate in most herds was due to indifference of owners when clinical manifestations were not apparent, or to a failure in following directions. One factor contributing to the failures was the purchasing of bred gilts irrespective of their status with respect to brucellosis. This common practice is the usual way of introducing the disease.

The feasibility of an eradication program based on segregation of young pigs is obvious when one considers the prolific reproduction of swine as compared with cattle. In swine raising, meat production is the ultimate objective, and therefore the salvage value of culled stock is considerable—a situation different from that with dairy cattle. The exception is found in valuable blood lines, but, under the program in question, such stock may be maintained in the herd with safety proportionate to the completeness of the segregation.

The data on the mature stock in both herds confirm the observations of Johnson and Huddleson[3] that agglutination titers tend to recede. In herd 1, although the infection was active, the titers of the heavily

infected foundation stock obviously tended to recede. Should such stock prove negative as far as the carrier state is concerned, there is evidence to suggest that acquired resistance may have an application in large herds under heavy exposure, where segregation is not practicable.

SUMMARY AND CONCLUSIONS

Data are presented to show that even in badly infected herds the majority of young pigs do not react at a titer of 1:25. On this principle, a program of eradication has been attempted in field herds; the data concern two of them. Although the disease was not eradicated from every herd, the freedom of the young pigs from infection showed that the program would be applicable in most situations and that the disease could be eradicated by segregating these pigs at weaning. The program is as follows:

1) Blood test the brood stock.

2) If infection is present, do not attempt to salvage nonreacting sows, but consider the unit as infected.

3) Segregate young pigs at weaning.

4) At breeding age, blood test and breed to a noninfected boar only those gilts negative at 1:25.

5) As replacements become available, cull infected stock.

References

[1]Cameron, Hugh S.: Brucellosis in Swine. I. The Interpretation of Low Titer Reactions in Experimental and Field Infections. A. J. Vet. Res., 4, (1943): 169-172.

[2]Howarth, J. A., and Hayes, F. M.: Brucellosis in the Swine Herd of the University of California. J.A.V.M.A., 78, (1931): 830-848.

[3]Johnson, H. W., and Huddleson, I. F.: Natural Brucella Infection in Swine. J.A.V.M.A., 78, (1931): 849-862.

Brucellosis in Swine
III. Studies on the Diagnostic Titer in the Individual

H. S. CAMERON, D.V.M., M.S., Ph.D., and P. A. CARLSON, D.V.M.
Davis, California

THE AGGLUTINATION test, like most serologic reactions, has certain limitations. It is effectively used in diagnosing brucellosis in a herd of swine, but is not considered reliable with respect to the individual. Although some of the discrepancies can be explained by the incubation period, wherein abortion may occur before agglutinins have appeared in the blood stream, many cases observed must be accounted for in another manner. Cameron, Hughes, and Gregory[1] have presented evidence showing the marked variation in agglutinin response in artificially infected unselected swine stock, tested at a minimum serum dilution of 1:25. In their subsequent work[2], *Brucella suis* was isolated from the lymph glands of negative pigs up to two months after exposure. Since comparable animals examined *post mortem* at later dates were negative serologically and bacteriologically, it would appear that the individual had overcome the infection. But such stock may, depending on the genital status, be a transient spreader.

Johnson and Huddleson[3], and Cameron[4] have noted that in swine a titer of 1:25 indicates exposure to Brucella. The literature, however, does not refer to the significance of still lower serum dilutions. The diagnostic titer in swine appears to have been arbitrarily established on the basis of experience with cattle. Huddleson[5] states: "Although the procedure of performing either the test-tube or rapid agglutination test on cattle serum may be used for other animals, the results cannot always be given the same interpretation. In many goats that have been found infected with Brucella, the agglutination test may be negative even after repeated tests. A negative test, therefore, does not always signify that infection is absent." Nonspecific agglutinins to Brucella are considered by many workers to be normal in cattle; on the other hand, they are not observed in the guinea pig or the mouse, hosts susceptible to Brucella. Conceivably, normal or nonspecific agglutinins for this organism are not present in swine, and an extremely low titer may be evidence of exposure. The work to be reported in this paper is based on the relation between the agglutination titer and the presence of *Br. suis* in the blood stream of artificially infected swine. The object was to establish a diagnostic titer applicable in the individual pig.

EXPERIMENTAL

The swine used in the experimental herd were grade and purebred pigs, the progeny of stock used in studies of genetic resistance to brucellosis. The genotype need not be considered here, but will be the subject of a later report. The first group consisted of 7 boars and 10 gilts, 12 to 14 weeks old. Of these, 4 boars and 6 gilts were infected orally with 5 cc. of a suspension containing 9 billion viable *Br. suis* per cc.; the remaining 3 boars and 4 gilts served as uninfected controls, but were kept in contact with the infected pigs. The second series consisted of 6 boars and 5 gilts, 14 to 17 weeks of age, all infected in a similar manner six weeks after the first group. The blood was tested for specific agglutinins and cultured for *Br. suis* before exposure and periodically thereafter. The hemoculture technique consisted of drawing 5 cc. of blood aseptically from the jugular vein into 15 cc. of 1 per cent tryptose broth or Kracke's medium. Complement was inactivated at 56 C. for fifteen minutes immediately following, and incubated at 37 C. Subcultures were made on beef infusion agar at weekly intervals for four weeks or until positive cultures were obtained. The tube technique was used for the agglutination test, with strain 19 *Brucella abortus* as an antigen. Since 1:25 and geometric progressions have been adopted as standards, the lower dilutions for this work conformed with that procedure. Thus they progressed in descending order as follows: 1:25, 1:12.5,

This project was coöperative with the United States Bureau of Animal Industry.

From the division of Veterinary Science, University of California, Davis, Calif.

1:6.25, 1:3.13, and 1:1.56. No evidence of negative pro-zones has appeared in these dilutions.

The first three tests were made at the 1:12.5 minimum dilution; then, when indicated by negative results, 1:6.25 was used. Later, when tests became negative, the two lower dilutions were employed. Reactions were not easily interpreted at these two latter high concentrations of serum. This difficulty was partially alleviated by using a washed antigen. Although the fluid became clear, the reaction was not considered positive unless agglutination was evident when the tube was agitated.

RESULTS

Table 1 shows the results of the agglutination tests and hemocultures in group 1 from exposure to 104 days. Blood reactions appeared eight days after exposure in 6 out of 10 animals; in 4 of these 6, hemocultures were positive for *Br. suis*. By the sixteenth day all 10 infected animals showed reactions in at least the 1:6.25 dilution. Positive hemocultures were infrequent after eleven days, although the organism was isolated from the blood of

TABLE 1—Agglutination and Hemoculture on Group 1 Pigs Inoculated at 12 to 14 Weeks of Age

Pig	Inoculum	0 Days Aggl.*	B.C.†	1 Day Aggl.	B.C.	4 Days Aggl.	B.C.	8 Days Aggl.	B.C.	11 Days Aggl.	B.C.
63F	Control....	—	—	—	—	—	—	—	—	—	—
65F	Control....	—	—	—	—	—	—	—	—	—	—
67F	Control....	—	—	—	—	—	—	—	—	—	—
69M	Control....	—	—	—	—	—	—	—	—	—	—
73F	Control....	—	—	—	—	—	—	—	—	—	—
74M	Control....	—	—	—	—	—	—	—	—	—	—
77M	Control....	—	—	—	—	—	—	—	—	—	—
61M	*Br. suis*....	—	—	—	—	—	—	+	+	+	+
62F	*Br. suis*....	—	—	—	—	—	—	—	—	—	—
64F	*Br. suis*....	—	—	—	—	—	—	+	+	+	—
66F	*Br. suis*....	—	—	—	—	—	—	+	—	+	I
68F	*Br. suis*....	—	—	—	—	—	—	+	+	+	—
70M	*Br. suis*....	—	—	—	—	—	—	+	+	+	I
75M	*Br. suis*....	—	—	—	—	—	—	—	—	—	—
76M	*Br. suis*....	—	—	—	—	—	—	+	—	+	—
78F	*Br. suis*....	—	—	—	—	—	—	—	—	—	—
79F	*Br. suis*....	—	—	—	—	—	—	I	—	+	I

Pig	Inoculum	16 Days Aggl.‡	B.C.†	18 Days Aggl.	B.C.	22 Days Aggl.	B.C.	31 Days Aggl.	B.C.	35 Days Aggl.	B.C.
63F	Control....	—	—	I	—	—	—	—	—	—	—
65F	Control....	—	—	—	—	—	—	—	—	—	—
67F	Control....	—	—	+	—	+	—	+	—	+	—
69M	Control....	—	—	—	—	—	—	—	—	—	—
73F	Control....	—	—			—	—	—	—	—	—
74M	Control....	—	—			—	—	—	—	—	—
77M	Control....	—	—	—	—	—	—	—	—	—	—
61M	*Br. suis*....	+	—	+	—	+	—	+	—	+	—
62F	*Br. suis*....	+	—	+	—	—	—	—	—	—	—
64F	*Br. suis*....	+	I	+	—	+	—	+	—	I	—
66F	*Br. suis*....	+	+	+	I	+	+	+	—	I	I
68F	*Br. suis*....	+	+	+	—	+	I	+	—	I	—
70M	*Br. suis*....	+	+	+	—	+	—	+	—	—	—
75M	*Br. suis*....	I	—	—	—	+	—	+	I	+	I
76M	*Br. suis*....	+	+	+	—	+	I	+	—	I	I
78F	*Br. suis*....	+	I			+	—	I	—	—	—
79F	*Br. suis*....	+	+	I	—	+	—	I	—	+	I

Pig	Inoculum	42 Days Aggl.‡	B.C.†	49 Days Aggl.	B.C.	56 Days Aggl.	B.C.	63 Days Aggl.	B.C.	70 Days Aggl.	B.C.
63F	Control....	—	—	—	—	—	—	—	—	—	—
65F	Control....	—	—	—	—	—	—	—	—	—	—
67F	Control....	—	—	I	—	+	—	I	—	I	—
69M	Control....	—	—	—	—	—	—	—	—	—	—
73F	Control....	—	—	—	—	—	—	—	—		
74M	Control....	—	—	—	—	—	—	—	—		
77M	Control....	—	—	—	—	—	—	—	—	—	—
61M	*Br. suis*....	+	I	+	I	+	—	+	I	+	I
62F	*Br. suis*....	—	—	—	—	—	—	—	—	—	—
64F	*Br. suis*....	I	—	—	—	I	—	—	—	—	—
66F	*Br. suis*....	I	—	I	—	I	—	I	—	I	—
68F	*Br. suis*....	I	—	—	—	+	—	I	—	I	—
70M	*Br. suis*....		—	I	—	—	—	—	—	—	—
75M	*Br. suis*....	+	I	+	—	+	I	+	—	I	—
76M	*Br. suis*....	+	+	+	—	+	+	+	I		
78F	*Br. suis*....	I	—	—	—	—	—	—	—		
79F	*Br. suis*....	+	I	+	I	+	I	+	—		

*Lowest dilution is 1:12.5 through fourth day, 1:6.25 thereafter; †Blood culture; ‡Lowest dilution is 1:6.25.

2 pigs thirty-five days after exposure. Of the 10 infected pigs, 6 showed positive hemocultures at some period during the observations, and 4 of the 6 were negative at the 1:25 dilution when cultures were positive.

Controls were negative on culture throughout the observation period, but a transitory reaction to the agglutination test appeared at the 1:6.25 dilution in 3 of the 7 pigs. These controls were in contact with the infected group.

Table 2 shows the results in the second group. These in general agree with the first. The titers, however, receded significantly faster than in the first series, perhaps because of a known variation in the genetic constitution of this group. Positive hemocultures, furthermore, were obtained on two occasions when the blood was negative for agglutinins at 1:6.25.

Table 3 shows the results of tests on group 2 at the lower dilutions. These tests were commenced thirty-five days after exposure, when most of the pigs were negative at 1:6.25 (*see* table 2).

DISCUSSION

According to these results, pigs may become infected with *Br. suis*, show a transitory bacteremia, and yet fail to react to the agglutination test at a serum dilution of 1:6.25. The absence of agglutinins before exposure and after recovery suggests that a reaction at this dilution in swine serums against Brucella antigen is specific and is evidence of exposure to that organism. The absence of agglutinins at 1:6.25 does not mean, apparently, that an exposed individual has remained healthy. Possibly, still lower titers must be resorted to for the early discovery of infection. Although table 3 suggests a specific reaction, further work is necessary on the two lower dilutions, for the purpose of improving the technique.

The experimental data concern only young pigs, but are supported by observations in a group of gilts, some of which were bred in an infected commercial herd. This group of 84 gilts was weaned from infected sows and segregated from the source of infection. At 7 to 9 months of age they were (except 1 gilt, which reacted at 1:25) negative at this minimum dilution. After two months more, 75 were tested; 57 failed to react at 1:6.25 or over; 1 had a titer of 1:12.5; and 7 had titers of 1:25 or over. On a test two weeks later, 20 of 45 had titers of 1:50 or higher. At least three abortions were observed, and others were known to have taken place; all of these reacted at high titers. Later,

TABLE 2—Agglutination and Hemoculture on Group 2 Pigs Inoculated at 14 to 17 Weeks of Age

Pig	0 Days		7 Days		14 Days		21 Days		28 Days		35 Days	
	Aggl.*	B.C.†	Aggl.	B.C.	Aggl.	B.C.	Aggl.	B.C.	Aggl.	B.C.	Aggl.	B.C.
71F	— — — —	—	— — — —	—	I — — —	—	+ — — —	—	+ — — —	—	I — — —	—
72F	— — — —	—	— — — —	—	I I + +	—	+ + + +	—	+ + + —	—	+ I — —	—
81M	— — — —	—	— — — —	—	I I + I	—	+ + — —	—	— — — —	—	— — — —	—
82M	— — — —	—	— — — —	—	I I I —	—	+ I — —	—	+ I — —	—	I — — —	—
83F	— — — —	—	I I — —	—	I I + +	—	+ + I —	—	+ I — —	—	— — — —	—
84F	— — — —	—	+ I — —	—	+ + — —	—	I — — —	—	I — — —	—	— — — —	—
86M	— — — —	—	— — — —	—	I + + I	—	+ + — —	—	+ — — —	—	— — — —	—
87M	— — — —	—	I — — —	—	— — — —	+	— — — —	—	I — — —	—	— — — —	—
88M	— — — —	—	I I — —	+	I I I —	—	+ I — —	—	+ I — —	—	I — — —	—
90M	— — — —	—	I I I —	—	I I I —	—	+ + — —	—	I I — —	—	— — — —	—
92F	— — — —	—	— — — —	—	I + + I	+	+ + — —	—	I I — —	—	— — — —	—

Pig	42 Days		49 Days		56 Days		63 Days		69 Days	
	Aggl.*	B.C.†	Aggl.	B.C.	Aggl.	B.C.	Aggl.	B.C.	Aggl.	B.C.
71F	— — — —	—	— — — —	—	— — — —	—	— — — —	—	— — — —	—
72F	— — — —	—	I* — — —		— — — —		— — — —		— — — —	
81M	— — — —	—	— — — —		— — — —		— — — —		— — — —	
82M	— — — —	—	— — — —		— — — —		— — — —		— — — —	
83F	— — — —	—	— — — —		— — — —		— — — —		— — — —	
84F	— — — —	—	— — — —		— — — —		— — — —		— — — —	
86M	— — — —	—	— — — —		— — — —		— — — —		— — — —	
87M	— — — —	—	— — — —		— — — —		— — — —		— — — —	
88M	I — — —	—	— — — —		+ I — —		I — — —		— — — —	
90M	— — — —	—	— — — —		I — — —		— — — —		— — — —	
92F	— — — —	+	— — — —		— — — —		— — — —		— — — —	

*Lowest dilution is 1:6.25; †Blood culture.

it was ascertained that on one unknown date early in January accidental severe exposure had occurred.

The removal of all animals showing titers of 1:6.25 or over failed to stop the spread of infection, since gilts negative at this minimum dilution had titers of 1:200 after two weeks. According to our present knowledge, therefore, brucellosis of swine cannot be handled on the basis of the individual, but must be considered a group problem under an eradication program where the blood test is involved. A study of herd history, particularly the trend in titers, must precede any attempt to salvage individuals from infected units. The evidence suggests that an initial titer of 1:6.25, or perhaps less, constitutes a recent exposure in a susceptible host. Such an individual is a potential spreader. On the other hand, a receding titer indicates recovery. The work of McNutt,[6] and unpublished data from this station, on the correlation of the titer and the presence of *Br. suis* in the lymph glands, show that a titer of 1:25 can be tolerated.

The purchase of bred gilts on a blood test of the individual, a common procedure and the origin of most outbreaks, would not assure the purchaser a noninfected animal. Emphasis should be placed on the status of the herd of origin, not on the test of the individual. Likewise if reactors are found in a group, the entire unit should be considered infected, and no attempt should be made to salvage nonreactors until successive tests have indicated that the infection is not actively spreading.

TABLE 3—Agglutination Tests at Serum Dilutions of 1:1.56 and 1:3.13 on Artificially Infected Pigs (see table 2)

Pig	35 Days A	35 Days B	42 Days A	42 Days B	49 Days A	49 Days B	56 Days* A	56 Days* B	63 Days A	63 Days B	69 Days A	69 Days B	78 Days A	78 Days B	85 Days A	85 Days B
71F	+	+	+	+	+	+	—	—	—	—	—	—	—	—	—	—
72F	+	+	+	+	+	+	—	—	I	—	I	I	I	—	I	I
81M	—	—	I	—	—	—	—	—	—	—	+	I	—	—	—	—
82M	I	I	I	—	I	—	—	—	—	—	—	—	—	—	—	—
83F	—	—	—	—	I	—	I	—	I	—	—	—	—	—	—	—
84F	I	I	+	—	+	I	—	—	+	—	—	—	—	—	—	—
86M	—	—	I	—	—	—	—	—	—	—	—	—	—	—	—	—
87M	I	I	—	—	+	+	—	—	+	I	—	—	—	—	—	—
88M	+	+	+	+	I	I	—	+	+	+	+	+	+	—	+	+
90M	I	I	—	—	I	—	—	I	I	I	+	—	—	—	—	—
92F	—	—	I†	—	+	—	—	—	I	I	I	—	—	—	—	—

A = 1:1.56; B = 1:3.13; *Washed antigen employed after 49th day; †Hemoculture positive.

SUMMARY AND CONCLUSIONS

The evidence presented indicates that a negative agglutination test even in serum dilution of 1:6.25 does not mean freedom from brucellosis in the individual pig in an infected herd. Positive blood cultures were obtained in experimentally infected pigs negative for agglutinins at 1:6.25.

The agglutinating titer must be interpreted with respect to the herd history, particularly the trend in titers. An initial titer of 1:6.25 is more dangerous than a receding one of 1:50.

In an eradication or purchase program involving the agglutination test, emphasis must be on the unit as a whole, not on the individual pig. If infection exists, the entire unit should be considered infected. Animals negative to the test should not be selected as noninfected from such a herd until repeated tests indicate that spread is not active in the group.

References

[1]Cameron, H. S., Hughes, E. H., and Gregory, P. W.: Studies on Genetic Resistance in Swine to Brucella Infection. Cornell Vet., *30*, (1940) : 218-222.

[2]Cameron, H. S., Gregory, P. W., and Hughes, E. H.: Studies on Genetic Resistance in Swine to Brucella Infection: II. A Bacteriological Examination of Resistant Stock. Cornell Vet., *31*, (1941): 21-24.

[3]Johnson, H. W., and Huddleson, I. F.: Natural Brucella Infection in Swine. J.A.V.M.A., *78*, (1931): 849-862.

[4]Cameron, Hugh S.: Brucellosis in Swine: I. The Interpretation of Low Titer Reactions in Experimental and Field Infections. Am. J. Vet. Res., *4* (1943): 169-172.

[5]Huddleson, I. F.: Brucella Infections in Animals and Man. The Commonwealth Fund, New York, 1943.

[6]McNutt, S. H.: Incidence and Importance of Brucella Infection of Swine in Packing Horses. J.A.V.M.A., *86*, (1935): 183-191.

Urine Excretion by Boars

W. W. GREEN, Ph.D.
St. Paul, Minnesota

DUKES[1] quotes Ellenberger and Scheunert, and states that the amount of urine excreted by pigs ranges from 2 to 6 liters per twenty-four hours with an average of 4 liters.

The urine samples cited in this paper were collected in connection with a study of urinary 17-ketosteroid excretion by boars. The boars used represented different lines of inbred swine being developed at the Minnesota Agricultural Experiment Station. (Winters *et al.*[2]). They ranged in age from 10 to 76 weeks and were either of spring or fall farrow. The fall farrowed boars were born in 1940 and were sampled from December, 1940, through the summer of 1941. The other group was farrowed in the spring of 1941 and assayed from the early summer of 1941 through the summer of 1942. There were, therefore, 2 groups approximately six months apart in age from which urine samples were secured during similar portions of separate years and simultaneously during the summer of 1941.

METHODS

Urine was secured by placing boars in crates with screened bottoms, with a collecting pan in such a position as to catch all the urine and yet allow the feces to fall free of the pan. Evaporation was controlled by the mechanical construction of the crate and also by a layer of toluene over the urine. As a sample accumulated, it was stored in a refrigerator at a temperature of 5 C. All urine samples were of that temperature at the time the volume was measured.

During the period of collection, the boars were fed a slop feed three times a day and offered water twice daily. While at liberty, all boars were confined in one pen and had free access to water and an adequate ration.

Sets of 4 boars were sampled at one time but 2 boars of any one line and similar age were never sampled simultaneously. Each individual was in the crate exactly forty-eight hours and all results are on the basis of the 48-hour samples. Each boar was placed in a collecting crate at ten to fourteen day intervals. Data were analyzed by methods given by Snedecor.[3]

RESULTS

A total of 486 samples of urine was collected. Of this total, there were 58 instances in which no urine was secured during the 48-hour period. The incidence of withholding urine was mainly confined to certain boars. Of the 44 boars used, 18 withheld urine at least once and all lines were represented. Those repeating the performance most frequently were confined to 7 boars; the number of samples withheld and the total number of attempts to collect were: 9 of 33, 8 of 28, C_2 line boars; 6 of 29, C_1 boar; 4 of 25, 7 of 22, M. L. boars; and 9 of 13, 4 of 22, Minn. No. 1 boars. Various drugs and mechanical methods were used in an attempt to force urination but the response was *nil*. The cause of the phenomena was not discovered unless it was the individual's reaction to the environment of the collecting crate.

Of the 428 samples yielding urine, the average quantity for the forty-eight hours was 4,357 cc., range 220 cc. to 15,600 cc. A total of 369 urine samples were assayed for 17-ketosteroids and most of the data of this paper are based on those samples. Urine samples not used resulted from a random choice of samples and their omission should not, therefore, have materially altered the results to be presented. The range in urine quantity of the 369 samples was, as before, 220 cc. to 15,600 cc.; the

Paper No. 2160, Scientific Journal Series of the Minnesota Agricultural Experiment Station. This study was conducted with animals produced in cooperation with the Regional Swine Breeding Laboratory, Ames, Iowa, Bureau of Animal Industry, U. S. Department of Agriculture.

From the Division of Animal and Poultry Husbandry, University of Minnesota, University Farm, St. Paul, Minnesota.

average was 4,287 cc. with a standard deviation of 2,299 cc.

Relationship of Age and Season to Urine Excretion.—Age of the boars apparently had more effect on the amount of urine excreted than did the season of the year. The parallelism in the trend of the average quantities of urine excreted shown in table 1 is rather striking in view of the fact that the samples were collected at the opposite seasons of the year.

Tables 1 and 2 give the average amounts of urine excreted by fall and spring farrowed boars of different ages and lines of breeding. Table 3 presents the coefficients of correlation of urine excretion with age and season units of the year. Season units (s) which designated the dates the samples were collected were made by dividing the year into five-day units beginning May 1. In order to give continuity to the years used, season unit 74 coincided in calendar dates with 1, 75 with 2, etc.

TABLE 1—Average Amount of Urine (cc.) Excreted per Forty-eight Hours by Fall and Spring Farrowed Boars

Season of Farrow	Age range (in weeks)			
	10-20	21-30	31-40	41-50
Fall*...	5421 (40)†	5689 (34)	4924 (35)	4951 (18)
Spring‡.	3437 (51)	4395 (54)	3100 (44)	2953 (27)

*Seven boars used in each age period.
†Number of samples given in parenthesis.
‡Number of boars used in each age period: 15, 14, 15, 9 for periods 10 to 20 to 41 to 50 weeks, respectively.

The fall farrowed boars excreted more urine at various ages than the spring boars (table 1). In order to test the significance of these differences, an analysis of variance was made with the degrees of freedom keyed as follows: variance due to differences, (a) between seasons, (b) among lines within seasons, (c) among boars within lines within seasons, (d) within boars within lines, and (e) total. When the mean square for seasons was tested by "lines within seasons" a seasonal difference was found to exist in the 10 to 20-week age group. Differences between seasons (tested by the mean square for "within boars within lines") existed at age groups of 21 to 30, 31 to 40, and 41 to 50 weeks, based on the boars used.

When possible seasonal effects on the trend in urine excretion were measured by comparing the change in amounts excreted between alternate calendar months for the fall and spring farrowed boars of similar ages, only one comparison indicated a difference.*

It will be noted from table 3 that the levels of significance were different for the correlations of urine quantity and age (r_{ux}) and urine quantity and season (r_{us}) of the fall and spring boars during the 31- to 40-week period; however, the correlations were not statistically different from one another.†

As correlations of urine quantity and age and urine quantity and season did not differ‡ for either the fall or spring groups, it may be concluded that "season" would not predict urine quantity more accurately than age.

The distribution of urine samples of different sizes throughout the year was tested by means of chi square. When the samples were coded into 17 classes of 500 cc. range, a highly significent chi square of 276 was secured and when the urine samples were grouped into four larger classes, 2,000 cc. per class, a highly significant chi square of 73 resulted. During January, February, and March more samples in the 6,000 to 8,000 cc. group were recovered than were expected, while fewer were found in June. More samples in the 2,000 to 4,000 cc. group were found in October than were expected. As the January to March data

*The boars were compared by aligning the samples of the fall farrowed boars for the month of December with those of the spring boars for the month of July. The average ages were 14 weeks for both groups. The difference in the amount of urine given by the spring boars between the months of July and September (September quantity minus July quantity) was then compared by an analysis of variance of differences with the companion difference of February quantity minus December quantity for the fall boars. The only comparison that showed a difference due to season (at the 5 per cent level) was the comparison of fall boars for the months of April to June and spring boars for November to January. The average ages were 31 and 40 weeks and 29 and 37 weeks, respectively, for the fall and spring boars.

†Compared by using Fisher's Z transformation (Treloar[4]).

‡Hotelling's method described by Baten[5].

TABLE 2—Average amount of Urine (cc.) Excreted per Forty-eight Hours by Boars of Various Lines and Season of Farrow

LINE AND SEASON OF FARROW	AGE RANGE (IN WEEKS)						
	10-20	21-30	31-40	41-50	51-60	61-76	AVERAGE
Spring 1941							
C_2	2850	6500	3201	2706	2595	3036	3481
M. L.	2950	3540	3548	2643	2066	4932	3280
Minn. #1	4406	4391	4682	3449	4581	3675	4197
B. S.	1280*	3750*	1400*	....	....	....	....
S. E.	....	3020*	2890*	....	....	....	....
C_1	....	1115*	1181	....	....	....	....
Fall 1940							
C_2	5023	5850	4653	3327	....	....	4713
M. L.	5779	5771	6215	5885	....	....	5913
Minn. #1 × C_1	5375	5248	3104	5520	....	....	4812

*These averages were based on few samples; other averages were based on 5 to 24 samples secured from 2 to 6 boars per line.

contained samples from boars of both spring and fall farrow, the discrepancies may be explained by the fact that the fall boars were in the age range of 21 to 30 weeks and were excreting urine at their highest levels. In June, the spring boars were young and were giving smaller quantities of urine because of the younger age. The abundance of 2,000 to 4,000 cc. samples secured in October was given by the spring boars, and it will be noted that October represented one of the months during their 31- to 40-week age period. Both spring and fall boars excreted less urine during that age period than in the preceding two periods. Thus the unequal distribution of urine samples of various quantities may be accounted for by age rather than by season of the year.

Line Differences in Urine Quantity.—Table 2 gives the average amount of urine excreted by boars of the various lines at different ages. Line differences were tested by means of an analysis of variance. The degrees of freedom were keyed as follows: variance due to differences, (a) between lines, (b) among boars within lines, (c) within boars within lines, and (d) total. As in the case of the analysis of variance for seasons, true line differences would be tested by comparing the variance of "between lines" by "boars within lines" and line differences based only on the boars used would be tested by "within boars within lines." True line differences were found for the spring farrowed boars for the ages of 21 to 30 weeks and 31 to 40 weeks. Line differences based on the boars used were secured at 10 to 20 weeks for the spring farrowed boars and at 31 to 40 weeks for the fall boars. Differences among boars within lines appeared at 41 to 50 weeks and 51 to 60 weeks for the spring boars and no differences were found in the fall boars.

DISCUSSION

The trend in the amount of urine excreted by the boars sampled was apparently more closely related to age than season of the year. Boars farrowed six months apart had the same type of curve representing the amount of urine excreted during the ages sampled. Only one minor difference was found in the trend of urine excretion between the fall and spring boars which might have been due to season of the year; however, season of the year did not predict the amount of urine excreted any better than did age, and all discrepancies from expected quantities could be explained on the basis of age of the boars rather than on the season of the year. The reason why the fall farrowed boars ex-

TABLE 3—Correlation Coefficients Between Urine Excretion by Boars (u) and Age (x) and Season Units of Year (s)

SPRING FARROWED BOARS			FALL FARROWED BOARS		
Age in weeks	r_{ux}	r_{us}	Age in weeks	r_{ux}	r_{us}
10-20	0.374*	0.437†	10-20	0.750†	0.743†
21-30	—0.016	—0.044	21-30	—0.158	—0.142
31-40	—0.114	—0.133	31-40	—0.433*	—0.429*
41-50	—0.357	—0.352	41-50	0.152	—0.494
51-60	—0.085	—0.079			
61-76	0.755*	0.708			

*Significant at the 5 per cent level ($P = < 0.05$).
†Significant at the 1 per cent level ($P = < 0.01$).

creted more urine than the spring boars was not ascertained.

Line differences in urine quantity would indicate a physiologic difference among the lines of swine. That the lines should differ in this respect was not surprising as these lines do differ in other physiologic traits. As differences in the quantity of urine excreted were not found among boars within lines before the ages of 41 to 50 weeks, it would indicate more strongly that the quantity was to some extent a line characteristic, i. e., all boars of a line tended to vary in similar ways at the same ages. It will be noted from table 2 that boars of the Minn. No. 1 line tended to excrete more urine than the C_2 spring 1941 or M. L. spring 1941 line boars and that the M. L. fall 1940 boars gave more than the C_2 fall 1940 or Minn. No. $1 \times C_1$ fall 1940 boars.

SUMMARY AND CONCLUSIONS

Forty-eight hour urine samples were recovered from boars of inbred lines of swine. The boars ranged in age from 10 to 76 weeks. They represented swine of both fall and spring farrow. The amount of urine in the 48-hour samples ranged from 0 to 15,600 cc., averaged 4,287 cc., and the standard deviation was 2,299 cc. Fall farrowed boars excreted more urine than spring farrowed boars. The trend in the quantity of urine excreted was apparently associated more with age than season of the year. Significant line differences were found in the amount of urine excreted. This indicated that the excretion of urine may be, in part, under genetic control.

References

[1]Dukes, H. H.: The Physiology of Domestic Animals. Comstock Publishing Co., Ithaca, N. Y. 1939.

[2]Winters, L. M., Comstock, R. E., Hodgson, R. E., Kiser, O. M., Jordan, P. S., and Dailey, D. L.: Experiments with Inbreeding Swine and Sheep. Minn. Agric. Exper. Sta. Bull. 364, (1943): 39.

[3]Snedecor, G. W.: Statistical Methods. Iowa State College Press, Ames, Iowa. 1940.

[4]Treloar, A. E.: Elements of Statistical Reasoning. John Wiley and Sons, Inc., New York, N. Y. 1939.

[5]Baten, W. D.: How to Determine Which of Two Variables is Better for Predicting a Third Variable. J. Am. Soc. Agron., 33, (1941): 695-699.

Sulfur Prophylaxis of Coccidiosis of Feeder Lambs

JOHN F. CHRISTENSEN, Ph.D.
Washington, D. C.

WITH THE coöperation of a large feeding establishment in central Nebraska, the Zoölogical Division of the Bureau of Animal Industry of the United States Department of Agriculture had an opportunity, during the feeding season of 1941-42, to conduct a large scale experiment on the prophylactic efficacy of ground crude sulfur* against naturally acquired coccidiosis of feeder lambs. The desirability of this experiment was suggested by the following considerations: (1) The coccidiostatic action and relatively low toxicity of elemental sulfur have been demonstrated in poultry; (2) the period of expectation of clinical coccidiosis in feeder lambs is so accurately known that prophylactic feeding of the chemical could be confined to a fairly well-defined period in the event it should prove effective as a preventive; and (3) the current high cost of sulfaguanidine, a drug having been demonstrated to have prophylactic value against clinical coccidiosis of feeder lambs, cattle, and poultry, together with the inherent difficulties in the control of the disease in feeder lambs by feeding practices, make some form of inexpensive chemical prevention extremely desirable, especially in areas where coccidiosis is a serious, recurrent problem.

EXPERIMENTAL PROCEDURE

The 1,400 lambs used in this experiment were taken at random from a single shipment originating at Artesia, New Mexico. Immediately after arrival at the site of the experiment, after three days *en route*, these lambs were penned in ordinary feedlots and given access to plenty of fresh water and alfalfa hay for approximately forty hours. The animals were then divided into 14 groups of 100 lambs each, and these groups were confined separately in adjacent feedlots, each measuring 32 ft. by 48 ft. and equipped with identical watering, feeding, and salting troughs. Immediately after confinement, the lambs were started on the experimental ration consisting of a mixture of chopped alfalfa and ground corn held together with molasses and water. The mixture was fed in low, open troughs, protected from excessive contamination by horizontal rails, between which the lambs had to reach to obtain feed. All groups of animals were fed all they would eat by keeping a considerable surplus of the feed available in the troughs at all times. The average susceptibility of these groups to coccidiosis was considered to be about equal, since all lambs were from the same shipment and received the same treatment prior to and during confinement in the experimental pens. The coccidial infections were acquired by natural means.

Eight groups of lambs were selected for sulfur feeding, so distributed that each sulfur-fed group was adjacent to at least 1 untreated control group. Three treated groups were fed ration mixtures containing 0.5 per cent (group 8), 1.0 per cent (group 4), and 1.5 per cent (group 14) sulfur by weight throughout the entire seventy-two days of the test. Two groups were given unmedicated meal for the first three days, then gradually increased proportions of sulfur were given until peaks of 1.5 per cent (group 6) and 3.0 per cent (group 16) were reached on the tenth day; these amounts were then maintained until the thirtieth day. Three sulfur-fed groups were fed, respectively, 2.0 per cent sulfur-meal mixture from the first to the thirty-ninth day (group 13), from the seventh to the thirtieth day (group 15), and from the tenth to the thirty-ninth day (group 17). In all cases, it was desired to have the lambs on their maximum sulfur dosage for at least a few days prior to and during the expected period for the development of clinical coccidiosis in the control lambs, which, in the writer's experience, is most likely to begin near the end of the second or during the third week of feeding. Six groups of lambs well distributed among these sulfur-fed groups were used as untreated controls (groups 3, 5, 7, 9, 10, and 11).

The fecal sampling method used in this experiment was designed to detect clinical coccidiosis. Therefore, only feces showing a tend-

From the Zoölogical Division, Bureau of Animal Industry, Agricultural Research Administration, United States Department of Agriculture, Washington, D. C. The author resigned May 30, 1942.

*The sulfur used in the experiment was a preparation of ground crude sulfur, approximately 150 mesh, obtained in a single shipment from a firm in Texas.

ency toward diarrhea, and thus suspected of being discharged from animals with clinical coccidiosis, were collected in the routine samplings from each pen. The procedure in collecting fecal specimens was as follows:

Small, approximately equal amounts of freshly discharged diarrheal or semidiarrheal feces were taken at random from the ground of each pen and pooled in a single sampling bottle. If the number of scouring lambs in a pen was less than 10, the pooled sample represented the composite of the feces from as many of these lambs as could be collected during the period of sampling. When the number of lambs discharging diarrheal or semidiarrheal feces totaled more than 10, the composite sample was taken from 10 well-distributed specimens. Samplings were made from each pen at three-day intervals, although observations to determine incidence of diarrhea and mortality were made daily. In the laboratory, the pooled fecal samples were thoroughly mixed with water and subjected to the whole feces dilution-counting method described earlier by the writer.[1] Treated and untreated groups of lambs were compared from the standpoints of incidence of diarrhea attributable to coccidiosis, coccidial oöcyst discharge in diarrheal and semidiarrheal feces, mortality from all causes, and percentage of fat lambs removed for shipment to market at the end of the experiment.

EXPERIMENTAL RESULTS

Incidence of coccidial diarrhea.—Significant differences were noted in the incidence of coccidial diarrhea between the untreated control lambs and the lambs receiving various percentages of sulfur in their ration (table 1). Lambs with clinical coccidiosis were readily distinguished from sulfur-fed or other control lambs, discharging diarrheal or semidiarrheal feces, by the gauntness associated with the acute stage of the infection, by the concentration of diarrhea and symptoms of coccidiosis into the third and fourth weeks of feeding, and by the association of high oöcyst production with diarrheal feces. The brownish to yellowish green, fetid, watery, or mushy feces, occasionally containing mucus or blood, or both, of the lambs with clinical coccidiosis were characteristically different from the mushy or diarrheal feces from lambs with temporary intestinal disturbances or from lambs showing the laxative effect of sulfur. Although the 6 control groups showed an average incidence of coccidial diarrhea of only 6.3 per cent, the contrast with the sulfur-fed lambs was sharp, since no case of coccidial dysentery was identified in the 8 groups of sulfur-treated animals.

The laxative effect of sulfur was directly proportional to the percentage of the chemical in the ration. In the groups fed sulfur for the entire seventy-two days, the inci-

TABLE 1—Results of Feeding Sulfur to Feeder Lambs

Group No.	Sulfur Production in Feed (%)	Sulfur Administrations Period of Administration (Days in Feedlot)	Coccidial Diarrhea (%)	Peak of Oöcyst Production (per cc. of pooled feces)	Mortality from All Causes (%)	Fat Lambs Removed after 72 Days (%)
3	—	—	8.0	25,000	6.0	42.0
5	—	—	6.0	137,000	4.0	35.0
7	—	—	8.0	35,000	3.0	22.0
9	—	—	4.0	120,000	3.0	42.0
10	—	—	6.0	192,000	5.0	43.0
11	—	—	6.0	42,000	8.0	41.0
Ave.			6.3	92,000	4.8	37.5
4	1.0	1-72	0	2,500	0.0	36.0
8	0.5	1-72	0	5,000	1.0	42.0
14	1.5	1-72	0	2,500	1.0	32.0
Ave.			0	3,300	0.7	36.6
6	0.5 1.0 1.5	4-6 7-9 10-30	0	5,000	2.0	34.0
16	1.0 2.0 3.0	4-6 7-9 10-30	0	5,000	8.0	28.0
Ave.			0	5,000	5.0	31.0
13	2.0	1-39	0	2,500	2.0	32.0
15	2.0	7-30	0	5,000	1.0	35.0
17	2.0	10-39	0	7,500	5.0	28.0
Ave.			0	5,000	2.7	31.6

Ave. = Average.

dence of diarrhea was almost negligible in the lambs on the 0.5 per cent sulfur feed, about 5 per cent in the lambs on the 1 per cent sulfur, and about 8 per cent in lambs on the 1.5 per cent sulfur feed. None of these groups, however, showed visible deleterious effects from these sulfur mixtures over the seventy-two day period. In lambs fed 2 per cent sulfur ration, the incidence of diarrhea fluctuated between 10 and 15 per cent, and after several weeks of sulfur feeding, occasional bloody feces were observed. Diarrhea in lambs fed 3 per cent sulfur ration ranged from 25 to 50 per cent, with corresponding increase in incidence of bloody discharges after continued feeding. Scouring due to sulfur feeding was characteristically different from that due to coccidiosis. The diarrheic or mushy feces from sulfur-fed lambs were fairly uniformly green to yellowish green and consisted for the most part of profuse puddles or heaps, differing in color, consistency, and odor from the feces of clinical coccidiosis. The bloody discharges in the sulfur-fed lambs were not due to coccidiosis, as demonstrated by the fact that the incidence of bloody discharge was directly correlated with the amount of sulfur fed. There was no relation between bloody discharges in these lambs and the clinical coccidiosis noted in the control lambs; moreover, the bloody discharges failed to reflect increase in the composite oöcyst count.

Oöcyst production.—There was a marked contrast between untreated control lambs and sulfur-fed lambs in multiplication of coccidia (tables 1 and 2). Diarrheal lambs in every untreated group showed significant increase in oöcyst discharge during the interval between the twelfth and twentieth days of the experiment (table 2), the peaks of discharge varying between 25,000 and 192,000 oöcysts per cc. of feces during this period. During the corresponding interval, no acceleration in oöcyst output was observed in any of the 8 sulfur-fed groups. On the contrary, there appeared to be an actual depression in the level of oöcyst discharge by these lambs, as was shown by the consistent failure of fecal specimens to show the presence of oöcysts by the dilu-

TABLE 2—Relation of Sulfur Feeding to Oöcyst Production in Feeder Lambs

Sampling Period (1942)	Sulfur-fed lambs																Control lambs											
	Group 4		Group 6		Group 8		Group 13		Group 14		Group 15		Group 16		Group 17		Group 3		Group 5		Group 7		Group 9		Group 10		Group 11	
	S	OC	S	OC	S	OC	S	OC	S	OC	S	OC	S	OC	S	OC	OC		OC		OC		OC		OC	
Jan. 10-12..	1.0	nc	—	nc	0.5	nc	2.0	nc	1.5	nc	—	nc	—	nc	—	nc	nc		nc		nc		nc		nc	
Jan. 13-15..	1.0	nc	0.5	nc	0.5	nc	2.0	nc	1.5	nc	—	nc	1.0	nc	—	nc	nc		nc		nc		nc		nc	
Jan. 16-18..	1.0	nc	1.0	nc	0.5	nc	2.0	nc	1.5	nc	2.0	nc	2.0	nc	—	nc	nc		nc		nc		nc		nc	
Jan. 19-21..	1.0	+	1.5	+	0.5	+	2.0	+	1.5	+	2.0	++	3.0	+	2.0	5,000	++		++		+		5,000		7,500		5,000	
Jan. 22-24..	1.0	+	1.5	+	0.5	5,000	2.0	+	1.5	+	2.0	+	3.0	+	2.0	+	+++		75,000		10,000		12,500		2,500		2,500	
Jan. 25-27..	1.0	+	1.5	+	0.5	++	2.0	+	1.5	+	2.0	5,000	3.0	+	2.0	+	12,500		75,000		7,500		120,000		+		42,000	
Jan. 28-30..	1.0	+	1.5	+	0.5	++	2.0	+	1.5	+	2.0	+	3.0	+	2.0	+	25,000		137,000		35,000		5,000		192,000		5,000	
Jan. 31-Feb. 2....	1.0	+	1.5	5,000	0.5	+	2.0	+	1.5	+	2.0	+	3.0	+	2.0	+	12,500		+		12,500		10,000		7,500		5,000	
Feb. 3- 5....	1.0	+	1.5	++	0.5	+++	2.0	+	1.5	+	2.0	+	3.0	5,000	2.0	+	5,000		7,500		5,000		+		+		12,500	
Feb. 6- 8....	1.0	+	1.5	+	0.5	+	2.0	7,500	1.5	+	2.0	+	3.0	nc	2.0	+	10,000		nc		12,500		7,500		+		5,000	
Feb. 9-11...	1.0	+	—	5,000	0.5	nc	2.0	+	1.5	+	—	+	—	+	2.0	+	+		nc		nc		nc		++		nc	
Feb. 12-14...	1.0	+	—	+	0.5	+	2.0	+	1.5	+	—	+	—	5,000	2.0	+	20,000		7,500		10,000		12,500		5,000		22,000	
Feb. 15-17...	1.0	+	—	nc	0.5	nc	2.0	+	1.5	+	—	nc	—	+	2.0	+	27,000		5,000		15,000		nc		nc		nc	
Feb. 18-20...	1.0	nc	—	nc	0.5	nc	—	nc	1.5	nc	—	nc	—	nc	—	nc	nc		nc		nc		nc		nc		nc	
Feb. 21-23...	1.0	+	—	+	0.5	+	—	+	1.5	+	—	++	—	++	—	5,000	+		+		+		7,500		++		5,000	
Feb. 24-26...	1.0	+	—	+	0.5	+	—	+	1.5	+	—	+	—	+	—	+	12,500		7,500		5,000		10,000		+		+	
Feb. 27-Mar. 1...	1.0	+	—	+	0.5	+	—	+	1.5	+	—	+	—	5,000	—	+	+		7,500		7,500		+		+		+	
Mar. 2- 4....	1.0	+	—	+	0.5	5,000	—	+	1.5	+	—	17,500	—	+	—	7,500	nc		nc		nc		nc		nc		nc	

S = Percentage of sulfur in feed; OC = Oöcyst count per cc. of pooled feces; + = Indicates fewer than 5,000 oöcysts per cc. of pooled feces; nc = No count made.

tion-counting technique used. The peak count recorded for group 15, sulfur-fed lambs in the March 2 to 4 sampling interval, occurred approximately three weeks after sulfur feeding was discontinued.

Total mortality.—No attempt was made to differentiate causes of death. Nevertheless, the differences in total mortality between untreated and treated lambs appeared to be significant. The average death loss in the 6 groups of untreated lambs during the seventy-two-day period was 4.8 per cent (groups 3, 5, 7, 9, 10, and 11), while during the same period, the corresponding loss was only 0.7 per cent in the 3 groups of lambs receiving 0.5, 1.0, and 1.5 per cent sulfur feed over the entire period (groups 4, 8, 14), and 3.7 per cent in the 5 groups of lambs that received graded or constant dosages of sulfur up to thirty or thirty-nine days of feeding (table 1). It is interesting to note, however, that out of a total of 20 deaths, noted in the 8 groups of sulfur-fed lambs during the seventy-two days of the experiment, only 4 occurred during the actual time that sulfur was incorporated in the ration.

Fat lambs assorted.—In spite of the greater incidence of coccidial dysentery, oöcyst output, and mortality, slightly more fat lambs were removed for shipment to market after seventy-two days of feeding from the untreated lambs than from the composite of the lambs fed an identical ration with sulfur added (table 1). The 3 groups of lambs fed 0.5 to 1.5 per cent sulfur over the entire period, however, yielded 36.6 per cent finished lambs as compared to a 37.5 per cent yield for the untreated lambs. Increase in percentage of sulfur above 1.5 per cent in the feed was associated with slower fattening rate, as shown by the fact that only 31 per cent of the animals fed 2.0 per cent or more of sulfur in the ration were ready for shipment after seventy-two days on the experimental feed.

DISCUSSION

With numerous large lamb feeders in the western feeding belt, particularly in the sugar beet areas of Colorado and Nebraska, outbreaks of coccidiosis occur with annoying frequency from year to year, and often produce heavy losses. With such feeders, therefore, the desire for preventing the disease is very great, especially since therapeutic measures have shown little promise of controlling outbreaks before substantial loss is experienced. That the disease can be kept under control by certain feeding practices has been fairly well demonstrated by workers at the Colorado Agricultural Experiment Station[2] and by the writer's investigations in Nebraska[3], but very often these practices are either not possible for the feeder in question or run counter to the economics of lamb fattening in a given area. Consequently, serious outbreaks of coccidiosis in the feedlots are to be expected with some frequency until an effective, yet economical method of chemical prophylaxis is available.

Steps in the direction of chemical prophylaxis of coccidiosis have already been taken in investigations with sulfaguanidine in feeder lambs[4]. Relatively small amounts of this drug, incorporated in the feed continuously for approximately three weeks from the day following massive experimental infection, effectively protected a small group of lambs from the disease. Since the infective inoculum given to these lambs probably far exceeded that ordinarily acquired in producing a severe natural outbreak of coccidiosis, it is believed that sulfaguanidine is a satisfactory prophylactic agent against this disease in lambs, but the present cost of the drug prevents its use for this purpose.

While showing definite promise in the directions indicated previously, the experiment reported here failed to show the maximum effectiveness of these dosages of sulfur in the prevention of clinical coccidiosis in feeder lambs, since the infections in the control lambs proved relatively mild. The chemical should now be tested against experimental infections of graded severity to determine its possible value against severe natural outbreaks of the disease. In the event that these experiments should continue to show prophylactic efficacy for

the chemical, further tests should then be made to determine the effect of continued feeding of sulfur on the presentability of the lamb carcass and the palatability of the flesh. The results presented here are intended as preliminary observations suggesting certain promise in the direction of sulfur prophylaxis of coccidiosis of feeder lambs and the desirability of additional work to determine the value of the procedure against heavy experimental and natural infections.

SUMMARY AND CONCLUSIONS

The experiment reported in this paper was conducted to determine whether ordinary elemental sulfur, an inexpensive and available chemical, might be used prophylactically to tide feeder lambs over the critical period for the development of clinical coccidiosis. The results point to the following significant conclusions:

1) Commercial flour sulfur, in portions ranging from 0.5 to 3.0 per cent of the total ration, effectively prevented the development of naturally acquired clinical cocidiosis in 800 lambs, although the infections in the 600 untreated control lambs were admittedly mild.

2) In portions greater than 1.5 per cent, the laxative effect of the sulfur was an objectionable feature, and weight gains did not compare favorably with gains in the control lambs.

3) No visible ill effects were noted in lambs after seventy-two days of continuous feeding of sulfur in amounts ranging from 0.5 to 1.5 per cent in the ration.

4) Lambs receiving 0.5 to 1.5 per cent sulfur in the ration continuously for seventy-two days showed remarkably low death rate from all causes, and compared favorably with untreated control animals in rate of fattening.

5) Feeding sulfur in these low percentages is decidedly practical, in view of the availability and low cost of this chemical.

References

[1]Christensen, John F.: The Effect of Copper Sulfate and Ferric Sulfate on Coccidial Oöcyst Output in Feeder Lambs. J.A.V.M.A., *96*, (1940): 478-480.

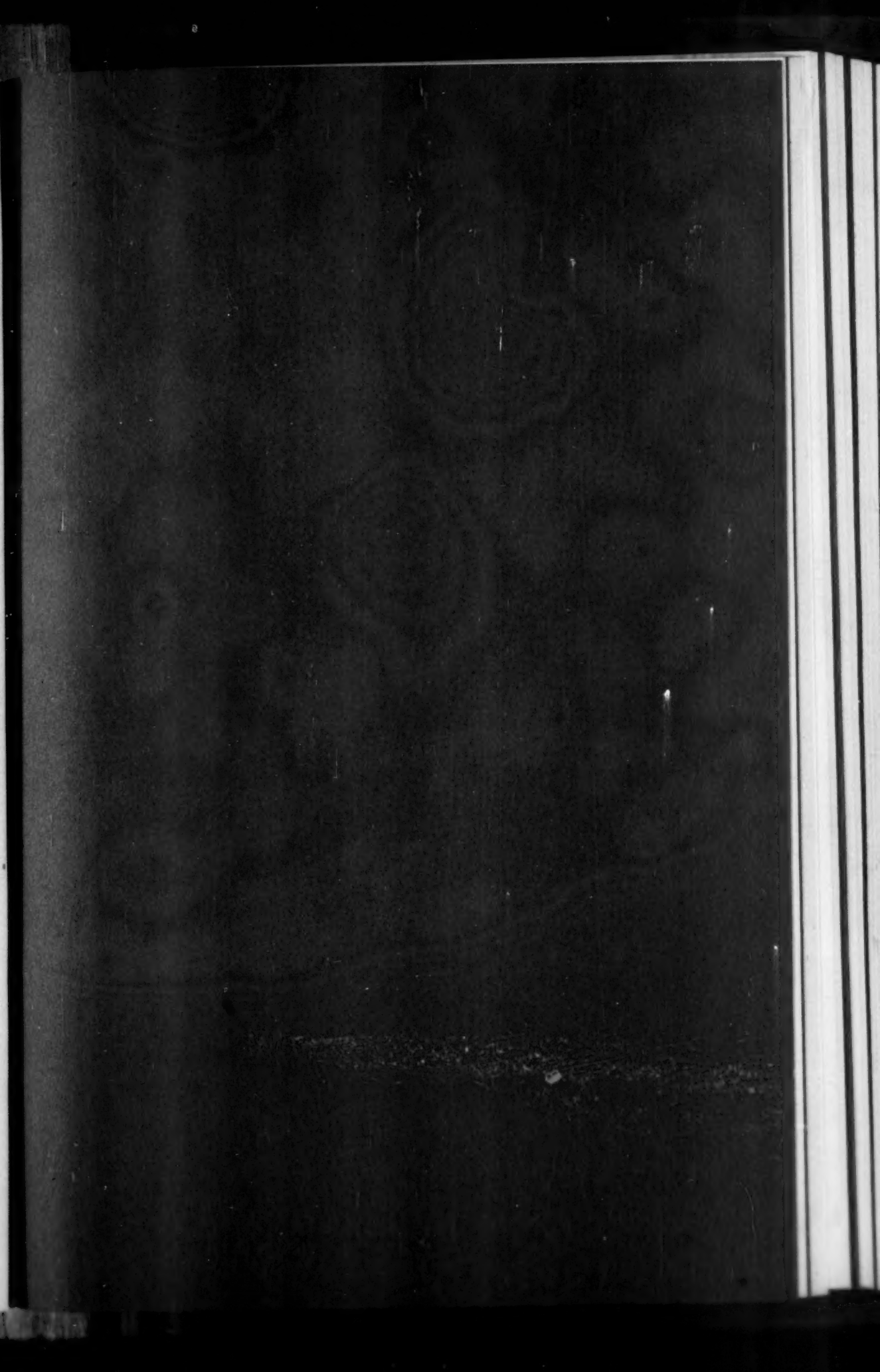
[2]Deem, A. W., and Thorp, Frank, Jr.: Coccidia and Coccidiosis in Feeder Lambs. J.A.V.M.A., *96*, (1940): 733-735.

[3]Christensen, John F.: The Source and Availability of Infective Oöcysts in an Outbreak of Coccidiosis in Lambs in Nebraska Feedlots. A. J. Vet. Res., *1*, (1940): 27-35.

[4]Christensen, John F., and Foster, A. O.: Further Studies with Sulfaguanidine in the Control of Ovine Coccidiosis. *Vet. Med.*, *38*, (1943): 144-147.

Notice to Subscribers

Last year, subscription notices for the ***AMERICAN JOURNAL OF VETERINARY RESEARCH*** *were mailed to all subscribers, including AVMA members, early in November in order to obtain an accurate estimate of the number of copies of the January (1944) issue needed, and thereby avoid waste of paper in an unnecessarily large over-run.*

Later in the year, dues notices were mailed to AVMA members, thus necessitating two remittances, in the event subscriptions to the ***RESEARCH JOURNAL*** *were desired. Many members objected to the dual remittances and requested that, in the future, they be enabled to send one check to cover both AVMA dues and renewal of their subscriptions to the* ***RESEARCH JOURNAL.***

Accordingly, this year, AVMA dues notices will be mailed early (about November 15), and will include an item for new or renewal subscriptions to the ***RESEARCH JOURNAL.*** *Your coöperation in making remittance promptly will be appreciated, even though the annual membership dues do not become payable until January 1.*